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## BRIEF COMMUNICATION



# Germline NPAT inactivating variants as cause of hereditary colorectal cancer

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Two independent exome sequencing initiatives aimed to identify new genes involved in the predisposition to nonpolyposis colorectal cancer led to the identification of heterozygous loss-of-function variants in *NPAT*, a gene that encodes a cyclin E/CDK2 effector required for S phase entry and a coactivator of histone transcription, in two families with multiple members affected with colorectal cancer. Enrichment of loss-of-function and predicted deleterious *NPAT* variants was identified in familial/early-onset colorectal cancer patients compared to non-cancer gnomAD individuals, further supporting the association with the disease. Previous studies in *Drosophila* models showed that *NPAT* abrogation results in chromosomal instability, increase of double strand breaks, and induction of tumour formation. In line with these results, colorectal cancers with *NPAT* somatic variants and no DNA repair defects have significantly higher aneuploidy levels than *NPAT*-wildtype colorectal cancers. In conclusion, our findings suggest that constitutional inactivating *NPAT* variants predispose to mismatch repair-proficient nonpolyposis colorectal cancer.

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## INTRODUCTION

Lynch syndrome is the most common inherited nonpolyposis colorectal cancer (CRC) syndrome, estimated to occur in 1 of every 280–400 individuals [1–3]. It is caused by germline pathogenic variants or epimutations in the DNA mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6*, *PMS2* and it is characterised by the presence of tumour MMR deficiency (dMMR) [4]. In contrast, the genetic basis of familial and/or early-onset nonpolyposis MMR-proficient (pMMR) CRC remains largely unexplained. Germline pathogenic variants in *RPS20* have been unequivocally linked to an increased risk of pMMR nonpolyposis CRC [5]. However, they explain a negligible number of unsolved families [6].

Here, *NPAT*, identified through two independent studies, emerges as a new candidate gene for pMMR CRC predisposition. Multiple pieces of evidence support its association with the disease, and we propose a potential carcinogenic mechanism for *NPAT*, resulting in high chromosomal instability.

## MATERIALS AND METHODS

This study includes two independent cohorts of genetically unexplained, pMMR familial and/or early-onset nonpolyposis CRC patients, which include 465 Spanish and 106 Dutch patients. GnomAD v2.1.1 non-cancer individuals were considered as control population [7]. Additional details on the cases

and controls studied, and a description of the methods used, which include exome sequencing and variant prioritisation strategies, *NPAT* mutational screening in validation cohorts, somatic second hit assessments and statistical analyses, are included in Supplementary Material and Methods.

## RESULTS AND DISCUSSION

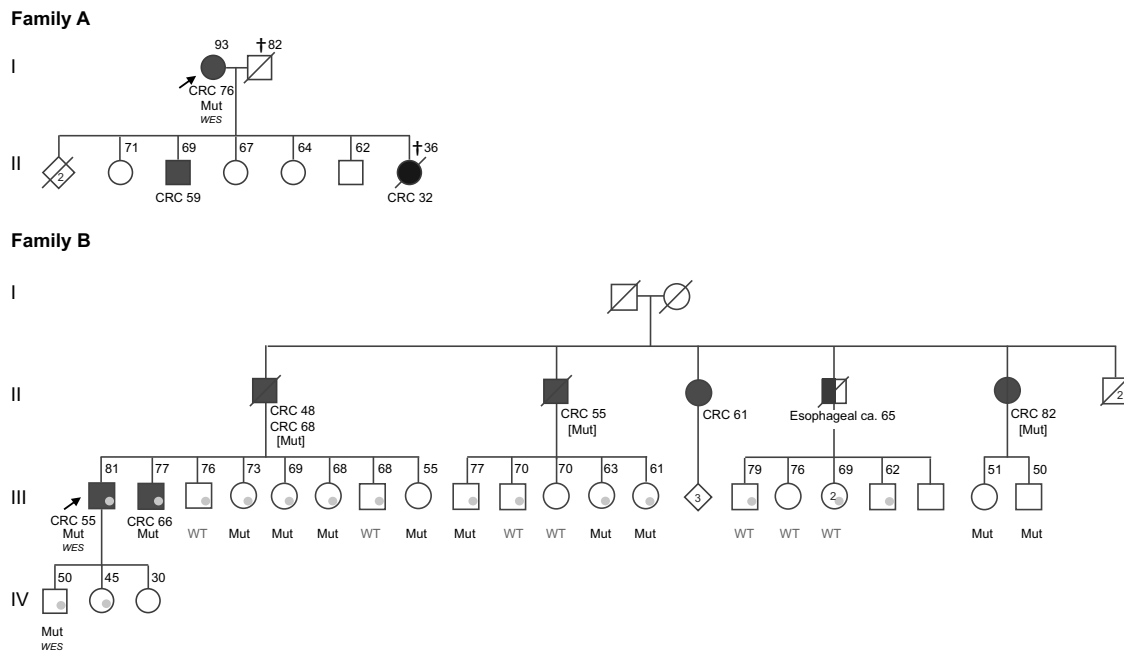
### Germline heterozygous *NPAT* variants in familial and/or early-onset CRC

In two pMMR CRC families of different geographic origins (Spain and the Netherlands) (Fig. 1), two independent exome sequencing analyses were conducted. No alterations or variants of unknown significance in known hereditary cancer genes were detected. Heterozygous loss-of-function variants in *NPAT* (NM\_002519.2: c.781G>T; p.(Glu261\*) and c.3452C>A; p.(Ser1151\*) [protein-coding sequence length: 1,427 amino acids] were identified in Family A and Family B respectively. Neither of these variants are reported in public population datasets (gnomAD v2.1.1, v3.1.2), Collaborative Spanish Variant Server (<http://csvs.babelomics.org/>), or Genome of the Netherlands (GoNL; accessed February 2024), except for one heterozygous c.3452C>A individual in 380,952 gnomAD v4.0.0 non-Finnish Europeans. The variant and gene prioritisation strategies that led to the selection of *NPAT* as candidate gene are detailed in Supplementary Material and Methods.

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**Fig. 1** Pedigrees of Family A (*NPAT* c.781G>T; p.(Glu261\*)) and Family B (*NPAT* c.3452C>A; p.(Ser1151\*)). Black symbol denotes CRC; grey dot inside the symbol, polyps identified in periodic colonoscopy screening; and half black symbol, any other tumour type. Whenever available, age at last follow-up or at death (+) is indicated in the upper right-hand side of the symbol. The cancer type and age at diagnosis, as well as the *NPAT* mutational status, are depicted below the symbol. ca. cancer, CRC colorectal cancer, Mut heterozygote for the corresponding *NPAT* variant, [Mut] obligate carrier, WT *NPAT* wildtype.

Six CRC-affected family members were heterozygous for the corresponding *NPAT* variant (Fig. 1). In these patients, (first) CRC was diagnosed at a mean age of 64 (range: 48–82), and no extracolonic tumours were reported. In Family A, only one of three CRC-affected members was available for analysis. No biological material from the CRC-affected offspring, diagnosed with CRC ages 59 and 32 could be obtained. In Family B, all five CRC-affected members were heterozygous or obligate heterozygous for *NPAT* c.3452C>A; p.(Ser1151\*), as well as 10 relatives without a CRC diagnosis (age range: 50–77). Due to the strong CRC family history, these 10 individuals had undergone periodic colonoscopy surveillance. In seven, premalignant lesions were identified and removed (mean age: 64.5), which may have prevented CRC occurrence. The remaining three had no colon tumour phenotypes at ages 50, 51 and 55. Eight additional family members, all cancer-free (age range: 68–79), had wildtype *NPAT* genotypes.

To further investigate the potential association of *NPAT* with CRC predisposition, we compared the frequency of loss-of-function and/or predicted deleterious alleles in *NPAT* in patients vs. controls. We evaluated *NPAT* mutational status in 464 Spanish and 105 Dutch additional familial/early-onset pMMR nonpolyposis CRC probands (1 CRC-affected individual per family) from the same cohorts as Families A and B, and analysed the publicly available data from 1006 unrelated CRC patients diagnosed before age 60 (data accessed via <https://canvar.icr.ac.uk/>) [8, 9]. We included 59,095 gnomAD (v.2.1.1) European non-Finnish, non-cancer individuals as controls. Loss-of-function and predicted damaging variants identified in cases and controls are listed in Table 1. In addition to the two truncating variants identified in Families A and B, two additional *NPAT* loss-of-function variants (c.1755del; p.(Glu586Asnfs\*30), and c.2635G>T; p.(Gly879\*)) and one missense predicted pathogenic variant (c.2549T>C; p.(Ile850Thr); REVEL: 0.544) were identified among the 1577 familial and/or early-onset CRC patients evaluated. Based on the three cohorts studied, 0.25% (4/1577) of familial/early onset CRC patients carried a heterozygous *NPAT* loss-of-function variant, or 0.32% (5/1577) when considering both loss-of-function and predicted deleterious (REVEL > 0.5) variants. Allele frequencies of *NPAT* loss-of-function variants in familial/early-onset CRC patients

(4/3,154; MAF = 0.13%) and controls (18/118,190; MAF = 0.015%) resulted significantly different, with an odds ratio (OR) of 8.3 (95% CI: 2.0–25.3;  $p = 0.0023$ ; Fisher's exact test). The differences were also observed when both loss-of-function and predicted damaging *NPAT* variants were considered (5/3154 (MAF = 0.16%) vs. 26/118,190 (MAF = 0.022%); OR = 7.2; 95% CI: 2.2–19.1;  $p = 0.0011$ ) (Table 1). The results of this enrichment test may be subject to bias due to the utilisation of different sequencing approaches in cases and controls, and among different cohorts of cases. A false positive result due to the modest sample size of the case group and the rarity of germline *NPAT* damaging variants might have also occurred. A validation study with larger cohorts is needed to confirm the effects observed.

Based on gnomAD v.4.0.0, the ratio of observed vs. expected loss-of-function variants in *NPAT* is low (36 observed vs. 100.2 expected = 0.36; 90% CI: 0.27–0.47), with a probability of loss of function intolerance (pLI) of 0.99. This suggests that *NPAT* is relatively intolerant to protein truncation, like other constraint genes that when mutated cause Mendelian diseases, including autosomal dominant hereditary cancer syndromes [10]. To date, no other Mendelian syndrome has been associated with pathogenic *NPAT* variants (<https://www.omim.org/>; May 2024).

Interestingly, *NPAT* shares its promoter with the cancer predisposition gene *ATM* (bidirectional 520-bp promoter). However, an effect on *ATM* expression of *NPAT* loss-of-function variants, which do not affect the promoter DNA sequence, seems unlikely.

#### ***NPAT* somatic status: evidence supporting haploinsufficiency**

The loss-of-function nature of the variants suggests a tumour suppressor nature for *NPAT*. No somatic second hit (LOH or mutation) was identified in the CRC developed at age 76 by the proband of Family A, however, somatic methylation of the *NPAT* promoter was not analysed and no other tumour samples from Family A or B were available for analysis.

We confirmed the expression of the (normal) major *NPAT* transcript in normal colon mucosa (CoTrEx v.2; <https://barcuvaseq.org/cotrex/>) and evaluated the presence and

**Table 1.** Germline *NPAT* (NM\_002519.2) damaging and predicted damaging (REVEL score >0.5) alleles identified in the non-cancer, non-Finnish European gnomAD v2.1.1 dataset, 1006 CRC patients diagnosed before age 60 (CanVar) [8, 9], the 465 patients of the familial and/or early-onset CRC Spanish cohort, and the 106 patients of the familial and/or early-onset CRC Dutch cohort.

| Cohort (#alleles)                                                       | <i>NPAT</i> Variant                 | Population <sup>a</sup> MAF | REVEL                          | # alleles                               |
|-------------------------------------------------------------------------|-------------------------------------|-----------------------------|--------------------------------|-----------------------------------------|
| Controls (118,190 alleles)                                              |                                     |                             |                                |                                         |
| GnomAD non-cancer<br>NFE_v2.1.1                                         | c.996dup; p.(Asp333*)               | 0.001%                      | –                              | 1                                       |
|                                                                         | c.945_946del; p.(Leu316Glyfs*14)    | 0.001%                      | –                              | 1                                       |
|                                                                         | c.4279G>T; p.(Glu1427*)             | 0.001%                      | –                              | 1                                       |
|                                                                         | c.4202_4205del; p.(Ile1401Argfs*15) | 0.001%                      | –                              | 1                                       |
|                                                                         | c.3953C>G; p.(Pro1318Arg)           | 0.001%                      | 0.608                          | 1                                       |
|                                                                         | c.3532_3535del; p.(Gln1178Lysfs*23) | 0.001%                      | –                              | 1                                       |
|                                                                         | c.3529del; p.(Arg1177Aspfs*25)      | 0.001%                      | –                              | 1                                       |
|                                                                         | c.3526G>T; p.(Glu1176*)             | 0.001%                      | –                              | 1                                       |
|                                                                         | c.334C>T; p.(Arg112*)               | 0.001%                      | –                              | 1                                       |
|                                                                         | c.3072-2A>T                         | 0.003%                      | –                              | 3                                       |
|                                                                         | c.2832_2836del; p.(Met945Argfs*14)  | 0.001%                      | –                              | 1                                       |
|                                                                         | c.2807C>A; p.(Ser936Tyr)            | 0.001%                      | 0.608                          | 1                                       |
|                                                                         | c.2789C>G; p.(Ser930Cys)            | 0.001%                      | 0.592                          | 1                                       |
|                                                                         | c.2717T>G; p.(Leu906*)              | 0.001%                      | –                              | 1                                       |
|                                                                         | c.2506del; p.(Ser836Glnfs*17)       | 0.001%                      | –                              | 1                                       |
|                                                                         | c.2465_2466del; p.(Ser822Cysfs*8)   | 0.001%                      | –                              | 1                                       |
|                                                                         | c.2437_2438del; p.(Leu814Phefs*6)   | 0.001%                      | –                              | 1                                       |
|                                                                         | c.2248_2251del; p.(Val750Profs*21)  | 0.001%                      | –                              | 1                                       |
|                                                                         | c.2027T>G; p.(Leu676*)              | 0.001%                      | –                              | 1                                       |
|                                                                         | c.2026_2027del; p.(Leu676Argfs*5)   | 0.001%                      | –                              | 1                                       |
|                                                                         | c.175_176del; p.(Leu59Aspfs*6)      | 0.001%                      | –                              | 1                                       |
|                                                                         | c.123_130del; p.(Cys41*)            | 0.001%                      | –                              | 1                                       |
|                                                                         | c.1133-2_1134del                    | 0.001%                      | –                              | 1                                       |
|                                                                         | c.1003+1G>A                         | 0.001%                      | –                              | 1                                       |
| <b>Damaging variants<sup>b</sup></b>                                    |                                     |                             | <b>Allele frequency</b>        | <b>18/118,190 (0.015%)</b>              |
| <b>Damaging<sup>b</sup> and predicted damaging variants<sup>c</sup></b> |                                     |                             | <b>Allele frequency</b>        | <b>26/118,190 (0.02%)</b>               |
| <b>Familial/eoCRC patients (3154 alleles)</b>                           |                                     |                             |                                |                                         |
| CanVar (2012 alleles)                                                   | c.1755delA; p.(Glu586Asnfs*30)      | –                           | –                              | 1                                       |
|                                                                         | c.2549T>C; p.(Ile850Thr)            | –                           | 0.544                          | 1                                       |
|                                                                         | c.2635G>T; p.(Gly879*)              | –                           | –                              | 1                                       |
| Spanish cohort (930 alleles)                                            | c.781G>T; p.(Glu261*)               | –                           | –                              | 1                                       |
| Dutch cohort (212 alleles)                                              | c.3452C>A; p.(Ser1151*)             | –                           | –                              | 1                                       |
| <b>Damaging variants<sup>b</sup></b>                                    |                                     |                             | <b>Allele frequency</b>        | <b>4/3154 (0.13%)</b>                   |
|                                                                         |                                     |                             | <b>OR (95% CI)<sup>d</sup></b> | <b>8.3 (2.05–25.33);<br/>p = 0.0023</b> |
| <b>Damaging<sup>b</sup> and predicted damaging variants<sup>c</sup></b> |                                     |                             | <b>Allele frequency</b>        | <b>5/3154 (0.16%)</b>                   |
|                                                                         |                                     |                             | <b>OR (95% CI)<sup>d</sup></b> | <b>7.2 (2.16–19.10);<br/>p = 0.0011</b> |

CI confidence interval, eoCRC early-onset colorectal cancer, MAF minor allele frequency, NFE non-Finnish Europeans, OR odds ratio.

<sup>a</sup>Population MAF corresponds to the minor allele frequency in the gnomAD v2.1.1, non-cancer, non-Finnish European dataset.

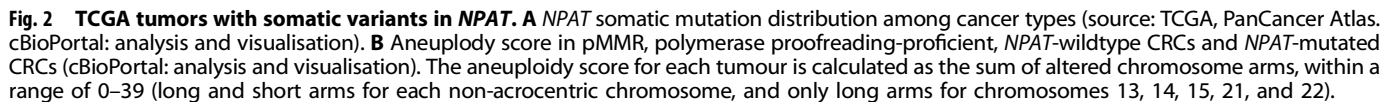
<sup>b</sup>Damaging variants include stop-gain and frameshift variants.

<sup>c</sup>Predicted damaging variants include canonical splice-site, start-loss and REVEL score >0.5 missense variants.

<sup>d</sup>P-values and ORs were calculated using Fisher's Exact Test for Count Data. Calculations were made in R console.

frequency of somatic mutations in *NPAT* among the different TCGA tumour types (source: [www.cBioPortal.org](http://www.cBioPortal.org)). The most frequently *NPAT*-mutated cancers were endometrial carcinoma (*NPAT* altered in 9.45% of 529 tumours), stomach adenocarcinoma (6.36% of 440), cutaneous melanoma (5.63% of 444), and CRC (4.55% of 594) (Fig. 2A), being colorectal, endometrial, gastric cancers the most representative tumours in known hereditary CRC syndromes (e.g. Lynch syndrome).

To find evidence supporting *NPAT* haploinsufficiency, as the potential absence of a second hit in the tumour of the Family A proband suggested, we further analysed the somatic status of the gene in the *NPAT*-mutated TCGA CRCs. Due to the mutational complexity of hyper- and ultra-mutated tumours, we studied the TCGA tumours with neither dMMR nor mutations in the exonuclease domains of polymerases  $\epsilon$  (*POLE*) or  $\delta$  (*POLD1*). These included one CRC with a stop-gain mutation, and five CRCs with



NPAT is a cyclin E/cyclin-dependent kinase 2 (CDK2) effector required for progression through the G1 and S phases of the cell cycle and for S phase entry, and is a key component of the histone locus body, involved in the transcription of canonical histone genes and in pre-mRNA processing [11, 12]. No association of

Studies in *Drosophila* showed that: i) *NPAT* (ortholog of *mxc* in *Drosophila*) mutations result in chromosomal instability caused by abnormal chromosome segregation promoted by diminished canonical histone levels [14]; ii) *NPAT* (*mxc*) knockdown causes increased rate of DNA double strand breaks [15]; and iii) several studies support the tumour suppressor role of *NPAT* (*mxc*) in this model organism [16–18]. Based on the evidence observed in *Drosophila* where *NPAT* (*mxc*) wildtype would prevent

chromosome instability, we analysed the characteristics of the six TCGA non-hyper/ultramutated CRCs with *NPAT* somatic variants. The analysis, performed through the cBioPortal website (<https://www.cbioportal.org/>), revealed a significantly higher aneuploidy level (number of altered chromosome arms) in these tumours than in *NPAT*-wildtype CRCs with the same characteristics ( $n = 50$ ) (Fig. 2B; TCGA tumours), supporting the observations in *Drosophila* and providing a potential explanation to the *NPAT*-driven carcinogenic mechanism. Unfortunately, the evaluation of chromosomal instability in the tumours of the patients heterozygous for *NPAT* deleterious variants could not be performed due to unavailability of tumour material.

## CONCLUSIONS

Our findings strongly suggest that inactivating germline pathogenic variants in *NPAT* are associated with an autosomal dominant predisposition to pMMR CRC. Available evidence, although still scarce, suggests that *NPAT* deficiency might lead to high chromosomal instability in tumours. Unlike most tumour suppressors, somatic inactivation of the second allele does not seem to be required, although additional evidence is needed to confirm haploinsufficiency. Further confirmation of the causal association of *NPAT* with CRC predisposition is needed before incorporating this gene into genetic testing in clinical practice.

## DATA AVAILABILITY

Data supporting the reported results may be found in the article or supplementary material. Additional data, analytic methods, and study materials will be made available to other researchers upon request.

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## AUTHOR CONTRIBUTIONS

LV, TvW and NFCCdM conceived and designed the project. MT, SAS, JV, GA, and PM under the supervision of LV, TvW and NFCCdM, performed the experiments, analysed, interpreted data and/or performed the statistical analyses. GP and DT performed/coordinated the exome-sequencing and data analysis in the Spanish cohort. DR performed next-generation sequencing analyses for the Dutch cohort. GC, MN, CMT, MEVL and HM provided samples, clinical data, and/or other type of resources to the project. LV, with the support of MT, SAS, TvW and NFCCdM wrote the manuscript. All authors were involved in writing and/or critically reviewing the manuscript and provided final approval for publication.

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## COMPETING INTERESTS

The authors declare no competing interests.

## ETHICS APPROVAL

The study received the approvals of the Ethics Committee of the Bellvitge Biomedical Research Centre (IDIBELL, Spain) (Protocol PR073/12), and the Medical Ethical Committee of the Leiden University Medical Centre (LUMC, The Netherlands) (Protocol P01.019). All individuals included in the study provided informed consent. The study adheres to the principles set out in the Declaration of Helsinki.

## ADDITIONAL INFORMATION

**Supplementary information** The online version contains supplementary material available at <https://doi.org/10.1038/s41431-024-01625-8>.

**Correspondence** and requests for materials should be addressed to Laura Valle or Tom van Wezel.

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