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Original Articles

Germline variation in O^6-methylguanine-DNA methyltransferase (*MGMT*) as cause of hereditary colorectal cancer

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ABSTRACT

Somatic epigenetic inactivation of the DNA repair protein *O6*-methylguanine DNA methyltransferase (MGMT) is frequent in colorectal cancer (CRC); however, its involvement in CRC predisposition remains unexplored. We assessed the role and relevance of *MGMT* germline mutations and epimutations in familial and early-onset CRC. Mutation and promoter methylation screenings were performed in 473 familial and/or early-onset mismatch repair-proficient nonpolyposis CRC cases. No constitutional *MGMT* inactivation by promoter methylation was observed. Of six rare heterozygous germline variants identified, c.346C > T (p.H116Y) and c.476G > A (p.R159Q), detected in three and one families respectively, affected highly conserved residues and showed segregation with cancer in available family members. *In vitro*, neither p.H116Y nor p.R159Q caused statistically significant reduction of MGMT repair activity. No evidence of somatic second hits was found in the studied tumors. Case-control data showed over-representation of c.346C > T (p.H116Y) in familial CRC compared to controls, but no overall association of *MGMT* mutations with CRC predisposition. In conclusion, germline mutations and constitutional epimutations in *MGMT* are not major players in hereditary CRC. Nevertheless, the over-representation of c.346C > T (p.H116Y) in our familial CRC cohort warrants further research.

1. Introduction

The activity of O^6-methylguanine DNA methyltransferase (MGMT), a DNA repair enzyme, consists in the removal of potentially mutagenic alkyl groups primarily from the O^6-position of guanine molecules. This type of DNA lesions can be caused both by endogenous [1] and/or exogenous alkylating agents [2]. MGMT activity is vital for genome integrity given that it prevents mismatch, replication and transcription

Abbreviations: APS, ammonium persulfate; CanVar, Cancer Variation Resource (https://canvar.icr.ac.uk/); CRC, colorectal cancer; ESP6500SI-V2 or ESP6500, NHLBI GO Exome Sequencing Project (http://evs.gs.washington.edu/EVS/); ESE, exonic splicing enhancer; ESS, exonic splicing silencer; FFPE, formalin-fixed paraffin-embedded; GnomAD, Genome Aggregation Database (http://gnomad.broadinstitute.org/); HNPCC, hereditary nonpolyposis colorectal cancer; HSF v3.0, Human Splicing Finder v.3.0; LOH, loss of heterozygosity; MAF, minor allele frequency; MGMT, *O6*-methylguanine DNA methyltransferase; MMR, DNA mismatch repair; MS-MCA, methylation-specific melting curve analysis; PBL, peripheral blood lymphocytes; RT-PCR, reverse transcription polymerase chain reaction; STS, sequence tagged site; TEMED, tetramethylethylenediamine; TBE, tris/borate/EDTA; Urea-PAGE, Urea polyacrylamide gel electrophoresis; WT, wildtype

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errors, which may lead to carcinogenic and apoptotic events [3,4].

Somatic alterations of *MGMT* have been reported in various tumor types including head and neck, non-small cell lung, brain, stomach, ovarian and colorectal tumors, being its inactivation mainly caused by promoter hypermethylation [5–8]. In particular, somatic epigenetic inactivation of *MGMT* has been reported as an early event in CRC [9–12], where it is known to be associated with *KRAS* and *TP53* mutations [5,13]. The methylation status of *MGMT* is a key prognostic/predictive factor for the treatment with alkylating drugs such as Temozolomide and Carmustine, especially in gliomas and metastatic colorectal cancer (CRC). In absence of methylation, MGMT is able to repair the cellular damage produced by alkylating agents, resulting in chemo-resistance. In contrast, MGMT silencing prevents the removal of alkyl groups, making the tumors sensitive to alkylating therapies [14–17].

The implication of *MGMT* germline mutations in hereditary cancer remains unexplored. Due to its role in DNA repair and its relevance in sporadic cancers, both features being characteristic of known hereditary cancer genes [18], we hypothesized that inherited pathogenic mutations and/or constitutional epimutations affecting *MGMT* might contribute to cancer predisposition, especially to CRC, where DNA repair genes are particularly relevant. We will focus our study on mismatch repair (MMR)-proficient familial and early-onset nonpolyposis CRC, whose genetic cause remains largely unknown [19].

2. Material and methods

2.1. Patients

With the aim of assessing the prevalence and effect of *MGMT* mutations in hereditary nonpolyposis CRC, we obtained peripheral blood DNA from 473 unexplained hereditary and/or early-onset nonpolyposis CRC patients (1 proband per family). All cases were MMR-proficient, i.e., their tumors showed microsatellite stability and/or expression of the MMR proteins MLH1, MSH2, MSH6 and PMS2. All tested individuals were affected with cancer, 96.2% with CRC. The mean age at cancer diagnosis was 49 (range: 16–82). Among the 473 studied families, 58 (12.2%) fulfilled the Amsterdam criteria, 385 (81.4%) the Bethesda guidelines, and 30 (6.3%), none of the established guidelines for hereditary nonpolyposis CRC. Detailed description of the hereditary nonpolyposis CRC cases included in this study is shown in Supplementary Table S1.

All patients, of European origin, were assessed at the Hereditary Cancer Program of the Catalan Institute of Oncology (Spain) between 1999 and 2017. Informed consent was obtained from the participants and all methods were performed in accordance with relevant guidelines and regulations. The study received the approval of the IDIBELL Ethics Committee.

2.2. DNA and RNA extraction

Germline DNA from peripheral blood samples was extracted using the FlexiGene DNA kit (Qiagen, Valencia, CA). Genomic DNA extraction from FFPE samples was performed using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The RNA used for splicing analysis was extracted from peripheral blood lymphocytes (PBLs) using a standard TRIzol®-based protocol (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Mutational screening of MGMT

Mutational screening of the five *MGMT* protein-coding exons and flanking sequences (± 20 base pairs) was performed by direct automated (Sanger) sequencing. Data was analyzed with SeqMan Pro v.13 (DNASTAR, Madison, WI, USA). Primers used for amplification and sequencing are shown in Supplementary Table S2.

2.4. Computational analyses

The predicted impact of missense variants at the protein level was analyzed using the *in silico* tool REVEL (variants with a score ≥ 0.5 were considered damaging), based on its good performance [20]. Additional predictions were provided by using SIFT [21] and PolyPhen-2 [22] (Supplementary Table S3). The potential effects on splicing were evaluated using Human Splice Finder v.3.0. Conservation scores PhyloP and PhastCons were obtained from the dbNSFP database (https://sites.google.com/site/jpopgen/dbNSFP). The effects on protein stability (Supplementary Table S4) were analyzed using PoPMuSiC v3.1 (http://dezyme.com), ERIS (https://dokhlab.med.psu.edu/eris/login.php), CUPSAT (http://cupsat.tu-bs.de), I-Mutant v2.0 (http://folding.biofold.org/i-mutant/i-mutant2.0.html), MAESTRO (https://biwww.che.sbg.ac.at/maestro/web/) and INPS-3D (http://inpsmd.biocomp.unibo.it/inpsSuite/default/index3D).

2.5. Loss of heterozygosity (LOH)

The presence of LOH in the tumors was determined by using eight different microsatellite markers: four sequence-tagged site (STS) markers, -D10S212, D10S1655, D10S217, and D10S1651,- spanning a region of 5.2 Mb, and four manually selected markers, two of which lie within the second intron of *MGMT* (MS1, MS2) and the other two immediately downstream of the gene (MS3, MS4). Fluorescently marked primers were designed to amplify the regions of interest (Supplementary Table S2). PCR amplification was performed in blood (normal) and matched tumor DNA, followed by capillary electrophoresis on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). LOH was considered if the intensity of any allele was reduced by 50% relative to the other allele after taking into account the relative allelic intensities in paired non-tumor DNA.

2.6. Culture of lymphocytes and splicing analysis

The potential effect on splicing caused by *MGMT* (NM_002412) c.333C > T (p.P111 =), c.346C > T (p.H116Y) and c.476G > A (p.R159Q), was evaluated in RNA extracted from PBLs of the corresponding carriers and controls cultured in presence and absence of puromycin, as previously described [23]. Retrotranscription was performed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, California, USA). RT-PCR was carried out to amplify the cDNA-specific *MGMT* sequence that included the potentially affected region (Primers in Supplementary Table S2). PCR products were run in a 1.5% agarose gel, visualized in a UV transilluminator and subsequently sequenced to check for splicing alterations.

2.7. Determination of constitutional and somatic MGMT promoter hypermethylation

Bisulfite conversion was carried out using the EZ-96 DNA Methylation Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's recommended protocol. *MGMT* promoter hypermethylation was determined by Methylation Specific Melting Curve Analysis (MS-MCA) in a LightCycler® 480 II (Roche, Basel, Switzerland), as described by Azuara et al. with slight modifications [24]. Samples were pre-amplified by PCR for 15 cycles using external primers. The resulting product was used as template for a 45-cycle PCR with internal primers, followed by melting curve analysis in the LightCycler® 480 II. The specific primers used are detailed in Supplementary Table S2. The technique was optimized using two control samples: 1) A blood DNA sample from a cancer-free control amplified by whole-genome amplification (GenomiPhi V2, GE Healthcare, Chicago, IL, USA), 0% methylated and used as negative control; and 2) CpG Methylated Jurkat Genomic DNA (New England Biolabs, Ipswich, MA, USA), 100% methylated and used as positive control. The sensitivity of the assay was

assessed by mixing known quantities of negative and positive controls in order to obtain 0%, 1%, 5%, 10%, 25%, 50% and 100% methylated samples (Supplementary Fig. S1). Methylation status was determined by comparing the melting curves obtained from cases versus controls. Direct bisulfite sequencing was used to confirm the MS-MCA results. Somatic *MGMT* promoter methylation was assessed by using both MS-MCA and direct bisulfite sequencing, including in the experiments the corresponding carriers' tumor and blood DNAs, as well as seven control DNAs obtained from FFPE normal colon tissue samples from *MGMT* wildtype individuals.

2.8. MGMT expression plasmids and transduction

MGMT cDNA was obtained from the MGMT pCMV6-AC-GFP plasmid (Origene, Rockville, MD, USA). The c.346C > T (p.H116Y) and c.476G > A (p.R159Q) variants were introduced into the wildtype sequence by Gibson Assembly [25], using the primers indicated in Supplementary Table S2. The inserts, MGMT-WT, MGMT-p.H116Y and MGMT-p.R159Q, were sub-cloned into the pLVX-ZsGreen1 plasmid (Clontech Laboratories, Mountain View, CA, USA), resulting in four different constructs: pLVX-ZsGreen1 (empty vector), pLVX-ZsGreen1_MGMT-WT, pLVX-ZsGreen1_MGMT-p.H116Y and pLVX-ZsGreen1_MGMT-p.R159Q. Ten micrograms of each plasmid were mixed with 7.5 µg of psPAX2 and 2.5 µg of pMD2.G plasmids in 1 ml of JetPRIME buffer and 50 µl of JetPRIME (Polyplus-transfection S.A., Illkirch, France). After 10 min of room temperature incubation, the transfection mix was added drop-wise to a 10-cm dish containing 10 ml of DMEM and Lenti-X 293T cells (Clontech Laboratories, Mountain View, CA, USA) at 80% confluence to produce lentivirus. After 72 h, the supernatant with high-titer lentiviral particles was recovered and 0.45-µm filtered. SW48 cells were incubated with 1.5 ml of concentrated viral supernatant supplemented with 10 µg/ml of polybrene (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) in six-well plates for 24 h. Cells high transduction efficiency was confirmed at the microscope by the presence of green fluorescence (ZsGreen1) and guaranteed by FACS sorting.

The SW48 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum, 1% penicillin and streptomycin, at 37 °C under a 5% CO2 atmosphere. MGMT protein expression was confirmed by Western Blot using the MGMT Antibody #2739 (Cell Signaling Technology, Danvers, MA, USA) at a concentration of 1:1000, following a standard protocol.

2.9. MGMT activity assay

The effect of p.H116Y and p.R159Q on MGMT repair activity was studied *in vitro* using a modified version of the MGMT Assay Kit MD0100 (Sigma-Aldrich, St. Louis, MO, USA). The modification was based on the use of fluorescently labelled oligonucleotides versus the radioactive labelling of the original protocol. Two 5′-HEX labelled oligonucleotides with identical genetic sequence, 5′-GAACTGCAGCTCCG TGCTGGCCC-3′, were used in this assay. The difference lay on the presence of an O^6-methyl-dG that blocks the PstI restriction site (CTGCA'G) in one of the oligonucleotides, while the non-methylated oligonucleotide served as positive control (unblocked PstI site). Incubation of the first (methylated) oligonucleotide with PstI results in no digestion and the presence of a unique 23-nt band when the product is run in a denaturing urea polyacrylamide gel (Urea PAGE). Incubation of the second (unmethylated) oligonucleotide results in two bands (8 and 23 nt).

The functional assay consisted of the incubation of the methylated oligo with the total protein (500 µg per reaction) extracted from the cell lysates obtained from the SW48 cell lines transfected with pLVX-ZsGreen1 (empty vector), pLVX-ZsGreen1_MGMT-WT, pLVX-ZsGreen1_MGMT-p.H116Y and pLVX-ZsGreen1_MGMT-p.R159Q. Subsequently, substrate extraction and precipitation was performed to purify the oligonucleotides and eliminate the proteins. DNA quantification using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) was carried out to ensure equal quantities of precipitated oligonucleotide for each MGMT variant-specific assay. The purified oligonucleotides were incubated with PstI (10 units per reaction) for 1 h at 37 °C, after which a digestion stop solution (90% Formamide, 20 mM EDTA) was added and the products denatured at 95 °C for 5 min. The products were run in a denaturing urea polyacrylamide gel (6 g Urea, 6.25 mL acrylamide/bis-acrylamide 40% solution, 1.25 mL TBE 10x, 5 µL TEMED and 125 µL APS 10%). Image acquisition was performed on a Typhoon FLA 9500 at 700 V and the results processed using the Image-Quant software (General Electric, Boston, MA, USA). Relative MGMT repair activity was calculated by dividing the intensity of the 8-nucleotide band by the sum of the intensities of the 23-nucleotide band and the 8-nucleotide one.

2.10. Statistical analyses

The frequencies of variants in *MGMT* in familial CRC cases and population controls were compared using Fisher's exact test. Bonferroni correction was applied for the association tests performed for the common variants. All tests were two-sided. The analyses were performed using R statistical software. MGMT activity differences between MGMT-WT and the different variants were compared using a *t*-test. The analysis was performed with GraphPad Prism 7 (GraphPad Software Inc., CA, USA), using data from three independent replicates. *P*-values below 0.05 were considered statistically significant.

3. Results and discussion

Based on the fact that somatic *MGMT* inactivation is mostly epigenetic [5–8], we hypothesized that constitutional *MGMT* promoter methylation may cause increased cancer risk, mimicking the constitutional *MLH1* epimutations found in early-onset and familial CRC patients [26,27]. However, no constitutional *MGMT* promoter methylation was detected in any of the 473 patients analyzed.

MGMT mutational screening of 473 unrelated MMR-proficient HNPCC cases identified 6 heterozygous rare ($MAF_{GnomAD} < 0.1\%$) variants -four missense (Supplementary Fig. S2) and two synonymous- in 9 unrelated families (Table 1). In addition to the rare variants (Supplementary Table S3), five common variants -two synonymous and 3 missense- were identified. No over-representation of these variants was found in the hereditary CRC cohort compared to cancer-free controls from the same geographical area (Supplementary Table S5). Likewise, a CRC case-control study in Spanish population showed no association with the disease (Supplementary Table S6).

No effect on splicing was predicted for c.471C > T (p.A157 =). Despite the predicted effect for *MGMT* c.333C > T (p.P111 =) (Human Splicing Finder v.3.0: New ESS, ESE disrupted), analysis of the carrier's RNA did not reveal splicing alterations (data not shown), thus discarding a deleterious effect.

Of the four identified missense variants, c.293A > C (p.N98T), located at a non-conserved residue (Supplementary Fig. S3) and predicted benign (REVEL score 0.062), was detected in an individual diagnosed with CRC at age 37 in the context of an Amsterdam-positive family. The microsatellite marker evaluation in search of tumor LOH revealed the presence of microsatellite instability (Supplementary Fig. S2), which led to the genetic analysis of a hereditary cancer multi-gene panel in the proband [28]. This approach allowed the identification of a pathogenic *POLE* mutation, c.881T > G (p.M294R), as the cause of the increased CRC risk in the family. The *POLE* mutation had been previously identified by our group in another hereditary cancer family, where hypermutation and the *POLE*-associated mutational signature [29] were detected in the carriers' tumors (unpublished data).

The c.319A > G (p.I107V) variant was identified in a 51 year-old

Table 1
Novel and rare (MAF < 1%) germline *MGMT* variants identified in 473 MMR-proficient hereditary and early-onset CRC probands. Population (non-Finnish European, non-cancer) MAF and phenotypic characteristics of the carrier families are indicated.

[a]*MGMT* variant [protein domain]	[b]European (non-Finnish) MAF (dbSNP)	[c]Evolutionary conservation (PhyloP/PhastCons)	Predictions: Function (REVEL), [d]structure and splicing (HSF v.3); RNA study	Proband's phenotype (age at diagnosis)/HNPCC criteria	Somatic 2nd hit (LOH/ mutation/promoter methylation)	Co-segregation
c.293A > C; p. N98T	0.00979% (rs369446171)	0.559/0.263	Benign (0.062) Structure destabilization (4/6) ESE disrupted	[e]CRC (37)/Ams	n.a.	n.a.
c.319A > G; p.I107V	n.a. (rs1473548584)	0.081/0.001	Benign (0.020) Structure destabilization (3/6) ESS created	[f]CRC (39)/Ams	no/no/no	n.a.
c.333C > T; p.P111 =	0.038% (rs41548114)	No amino acid change	n.a n.a. ESS created, ESE disrupted. RNA study: No splicing alteration	Fam 1: CRC (40)/Beth Fam 2: CRC (52)/No criteria	n.a. n.a.	n.a. n.a.
c.346C > T; p.H116Y [DNA Methyltransferase]	0.002% (rs199734815)	5.101/1	Benign (0.304)[g] Structure destabilization (4/6) ESS created, ESE disrupted RNA study: No splicing alteration	Fam 1: CRC (49)/Beth (Fig. 1B)	n.a.	Carriers: Sister (Breast ca., 49); son (cancer-free, 40) Non-carriers: Children (cancer-free, 38 and 48)
				Fam 2: CRC (62)/Beth (Fig. 1C)	no/no/no.	n.a.
				Fam 3: CRC (44)/Beth (Fig. 1D)	no/no/n.a.	n.a.
c.476G > A; p.R159Q [DNA Binding]	0.02% (rs3750824)	5.669/0.999	Benign (0.447)[g] Structure destabilization (1/6) ESE disrupted. RNA study: No splicing alteration	CRC (38)/Ams (Fig. 1A)	Tumor proband: no/no/no Tumor mother: n.a./no/no Tumor aunt: n.a./no/no	Carriers: Mother (CRC, 47); maternal aunt (End. ca. 67) Non-carriers: Father (Skin ca, 69; CRC, 74); brother (Lung ca., 48)
c.471C > T: p.A157 =	n.a. (rs202113261)	No amino acid change	n.a n.a. No predicted alterations	CRC (37)/Ams	n.a	n.a

Abbreviations: Ams, Amsterdam criteria; Beth, Bethesda criteria; Ca., cancer; CRC, colorectal cancer; End.ca., endometrial cancer; HNPCC, hereditary nonpolyposis CRC; HSF v.3, Human Splicing Finder v.3; MAF, minor allele frequency; n.a., not available; n.r., not reported.

[a] (NM_002412; GRCh37).

[b] Source: Genome Aggregation Database (GnomAD) v.2.1. Non-cancer subpopulation.

[c] PhyloP score (values between −14 and + 6): Sites predicted to be conserved are assigned positive scores. PhastCons score (values between 0 and 1): It reflects the probability that each nucleotide belongs to a conserved element, based on the multiple alignments of genome sequences of 46 different species (the closer the value is to 1, the higher the probability for the nucleotide to be conserved).

[d] Structure destabilization prediction calculated with PoPMuSiC v3.1, ERIS, CUPSAT, I-Mutant v2.0, MAESTRO and INPS-3D (Supplementary Table S4 for detailed information). Between brackets, number of programs predicting structure destabilization.

[e] Phenotype explained by *POLE* pathogenic mutation.

[f] Also carrier of a functionally relevant mutation in *BRF1* (c.35C > T; p.T12M).

[g] p.H116Y and p.R159Q were predicted deleterious by SIFT and PolyPhen-2 (Supplementary Table S3).

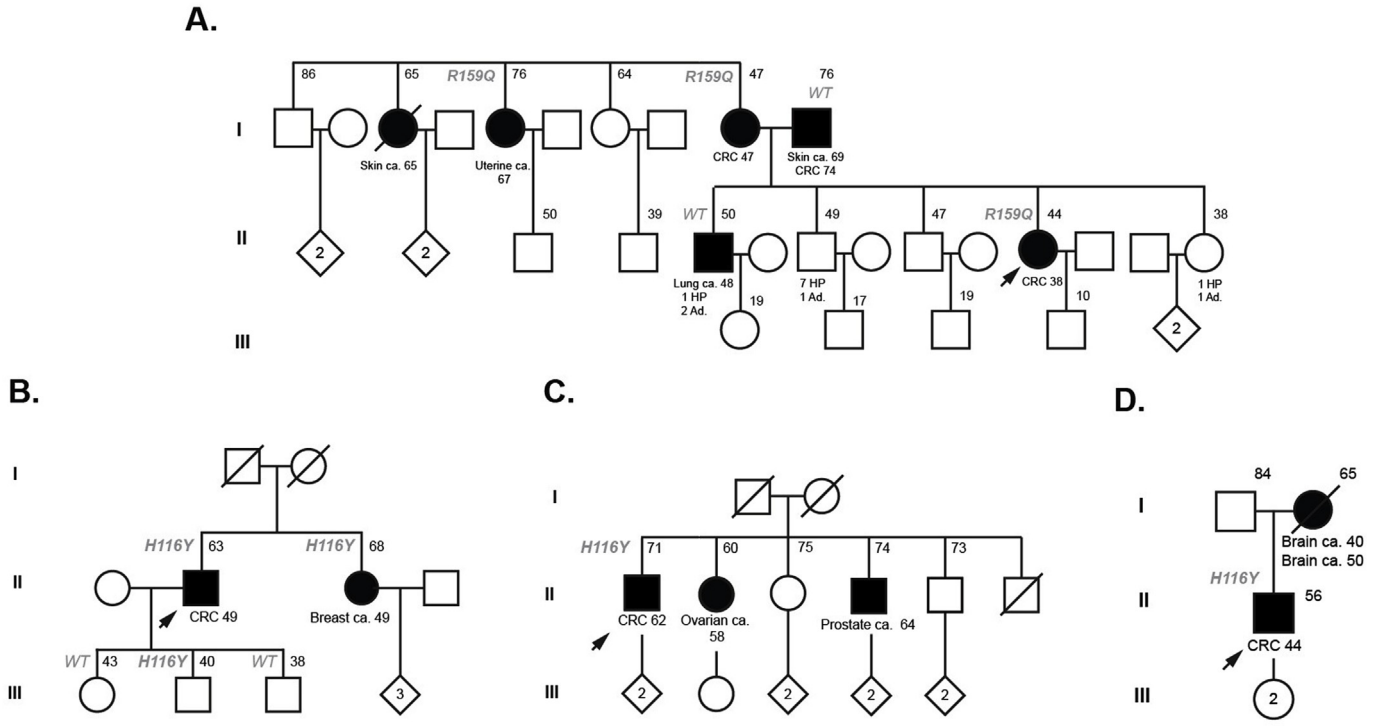

Fig. 1. Pedigrees of the families carrying *MGMT* p.R159Q and p.H116Y. Filled black symbol, affected with cancer; black arrow, index case. Ages at information gathering or at death, when available, are indicated on the top-right corner, and ages at cancer diagnosis, after tumor type. Mutation carriers are indicated with the corresponding variant, and non-carriers, as WT. Abbreviations: ca, cancer; CRC, colorectal cancer; HP, hyperplastic polyp; Ad., adenoma.

woman diagnosed with CRC at age 39, also from a family fulfilling the Amsterdam I criteria. No biological material could be obtained from either her mother or her maternal uncle; both diagnosed with CRC at ages 67 and 52, respectively. The variant, predicted benign (REVEL score: 0.02), was located at a non-conserved residue (Supplementary Fig. S3). No somatic second hit was identified in the proband's colon tumor (Table 1; Supplementary Fig. S2). These findings, together with the fact that the proband also carried a functionally relevant mutation in *BRF1* (c.35C > T; p.T12M), a recently reported candidate hereditary CRC gene [30], do not support a pathogenic effect for *MGMT* c.319A > G (p.I107V).

The other two mutations, c.346C > T (p.H116Y) and c.476G > A (p.R159Q), identified in three and one families, respectively (Fig. 1), affected highly conserved residues (Supplementary Fig. S3). Both were predicted benign by REVEL (scores: 0.304 for p.H116Y, and 0.447 for p.R159Q) (Table 1). Despite this prediction, mutations affecting residue 159, the arginine finger located in the DNA binding domain, had been reported in the literature as responsible of the loss of the DNA repair activity (p.R159G) and the reduction of activity towards methylated DNA by over 1000 fold (p.R159A and p.R159D) [31,32]. Likewise, histidine 116 is located within the methyltransferase domain, being a Zinc-binding residue (Supplementary Fig. S4).

The c.346C > T (p.H116Y) variant was found in three unrelated CRC patients. All three families fulfilled the Bethesda guidelines for hereditary nonpolyposis CRC (Fig. 1B, C and D). Cosegregation could be performed in one family (Fig. 1B), where the proband's sister, affected with breast cancer at age 49, and one of his three cancer-free descendants (ages 38–43), resulted carriers. Second hit analyses could be performed in the colon tumor samples of two c.346C > T carriers (probands of the families represented in Fig. 1C and D); no *MGMT* somatic LOH or mutation were detected in any of them (Supplementary Fig. S2). *In vitro*, no statistically significant reduction in MGMT activity was detected for p.H116Y when compared to the wildtype control (p-value = 0.2114) (Fig. 2). Of note, the results of the *in vitro* assay should be nevertheless interpreted with caution, as we have no data about the sensitivity of the assay to detect incomplete reduction of MGMT activity. The variant, present in 3 families in our study (0.63%), was not found in 1006 exomes of familial/early onset CRC cases (CanVar; https://canvar.icr.ac.uk/). Considering the two studies altogether, the frequency of p.H116Y in (European) familial CRC is 3/1479 (0.20%); significantly higher than the frequency observed in non-Finnish European cancer-free population (2/51,079; 0.0039%; source: GnomAD v.2.1) (p = 0.0002131, Fisher's exact test). Although all (or most) cases and controls are of European origin, they are not properly matched, which may have caused the observed imbalance. When focused on Spanish population, no carriers of p.H116Y were found among 1613 unrelated cancer-free Spanish individuals (CIBERER Spanish Variant Server, cancer group excluded; http://csvs.babelomics.org/) compared to the 3 carriers identified in the hereditary CRC cohort (3/473 cases vs. 0/1613 in controls; (p = 0.0116; Fisher's exact test).

The c.476G > A (p.R159Q) variant was identified in a 44 year-old woman diagnosed with colon cancer at age 38, and in her mother and maternal aunt, diagnosed with CRC at age 47 and endometrial cancer at age 67, respectively (Fig. 1A). The proband's father, diagnosed with skin and colon cancers at ages 69 and 74, and her brother, diagnosed with lung cancer, were not carriers of the *MGMT* variant. No somatic second hits were detected in the tumors from the three c.476G > A carriers (Supplementary Fig. S2). The *in vitro* MGMT activity assay showed no statistically significant differences for p.R159Q when compared to wildtype MGMT (p-value = 0.6236) (Fig. 2), *a priori* supporting the benign nature of the variant. In fact, p.R159Q was not found at higher frequency in familial CRC cases (1/473 families from the current study *plus* 0/1006 familial/early-onset CRC cases available at https://canvar.icr.ac.uk/; total 1/1479 (0.067%)) compared to non-Finish European Population (GnomAD) (27 heterozygotes (0.4%) and 1 homozygote in 63,353 individuals), confirming the lack of association with CRC. This lack of association was also found when comparing only Spanish individuals (1/473 cases vs. 1/1613 cancer-free controls; source http://csvs.babelomics.org/; p = 0.4022).

Finally, we evaluated the overall presence of germline *MGMT*

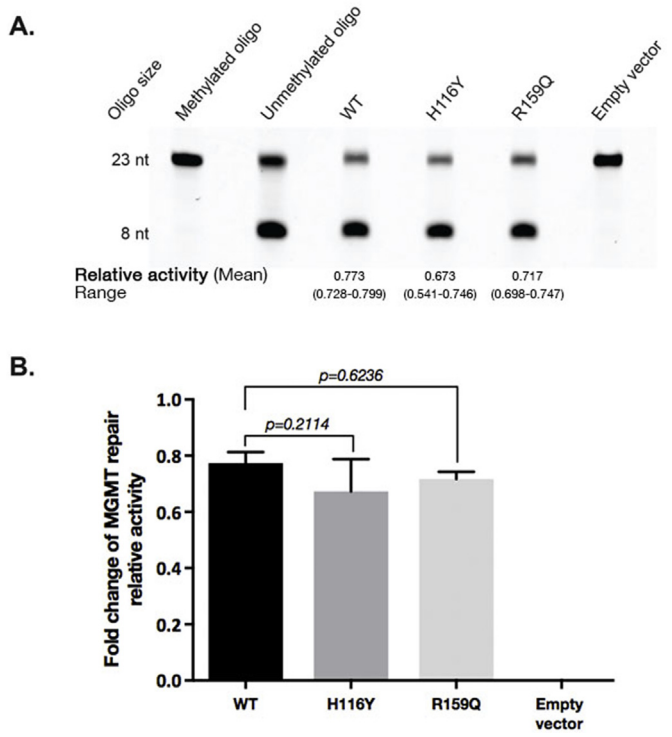

Fig. 2. MGMT repair activity assay. (A) Results from a single MGMT assay by using protein extracts prepared from SW48 cell line transfected with the *MGMT* missense variants (p.H116Y and p.R159Q), empty vector, and wildtype (WT). The amount of non-repaired substrate migrates as a 23-nucleotide (nt) oligonucleotide (upper band), while the repaired substrate migrates as an 8-nt oligonucleotide (lower band). Relative activity is calculated for each sample by dividing the intensities of the 8-nt band by 23-nt plus 8-nt bands. The indicated value of relative activity corresponds to three independent experiments (mean and range). **(B)** Graph showing the results of the MGMT activity assay, including the mean (bars) and standard deviation (SD) from the three experiments.

mutations in cancer-free population and familial CRC, in order to provide a definitive answer about the association of *MGMT* germline mutations with CRC predisposition. To do that, we analyzed: i) the publicly available data in the NHLBI GO Exome Sequencing Project, which includes data from 4300 Caucasians (ESP6500SI−V2 (not related to cancer), European American population); ii) the 1006 exomes of familial/early onset CRC cases analyzed by Chubb et al. [33] and available at https://canvar.icr.ac.uk/; and iii) the results obtained from the 473 familial/early-onset CRC unrelated cases analyzed in our study. We considered all variants with a population $MAF_{GnomAD} < 1\%$ that were stop-gain, frameshift, affecting canonical splice sites (reference transcript NM_002412.3), or missense variants predicted deleterious by REVEL (cutoff score: 0.50). Based on these criteria, three loss-of-function mutations in *MGMT* were identified among the 4300 Caucasians included in NHLBI GO Exome Sequencing Project (0.07% controls), and no germline (predicted) pathogenic *MGMT* mutations were identified either in our study (0/473 familial CRC) or in the familial CRC cases published by Chubb et al. (0/1006), suggesting no association of germline *MGMT* mutations with CRC predisposition. These data should be interpreted with caution because, although all from European populations, cases and controls are not properly matched.

4. Conclusions

Our findings suggest that, overall, germline mutations and epimutations in *MGMT* are not major colorectal cancer predisposing factors. Nevertheless, the over-representation of c.346C > T (p.H116Y) in familial CRC warrants further research to confirm or discard its association with cancer predisposition and if confirmed, estimate the associated risks to assess its value for genetic testing and counselling.

Conflicts of interest

The authors declare no conflict of interest.

Authors' contributions

LV and ME supervised the study. LV and ME contributed to the study concept and design. SB, CM, PM, FS, PLA, MPS, TP and MN performed experiments and/or acquired data. SB, CM, PM, TP, MP and LV contributed to the analysis and interpretation of data. MN, MP, GC and LV contributed to the acquisition of samples and clinical data. SB and LV drafted the manuscript. All authors critically reviewed the manuscript for important intellectual content and approved its final version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.01.019.

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