**Human Cancer Biology**

**Analysis of the Oxidative Damage Repair Genes NUDT1, OGG1, and MUTYH in Patients from Mismatch Repair Proficient HNPCC Families (MSS-HNPCC)**

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**Abstract**

**Purpose:** Several studies have described molecular differences between microsatellite stable hereditary nonpolyposis colorectal cancer (MSS-HNPCC) and microsatellite unstable Lynch syndrome tumors (MSI-HNPCC). These differences highlight the possibility that other instability forms could explain cancer susceptibility in this group of families.

The base excision repair (BER) pathway is the major DNA repair pathway for oxidative DNA damage. A defect in this pathway can result in DNA transversion mutations and a subsequent increased cancer risk. Mutations in MUTYH have been associated with increased colorectal cancer (CRC) risk while no association has been described for OGG1 or NUDT1.

**Experimental Design:** We performed mutational screening of the three genes involved in defense against oxidative DNA damage in a set of 42 MSS-HNPCC families.

**Results:** Eight rare variants and 5 frequent variants were found in MSS-HNPCC patients. All variants were previously described by other authors except variant c.285C>T in OGG1. Segregation studies were done and in silico programs were used to estimate the level of amino acid conservation, protein damage prediction, and possible splicing alterations. Variants OGG1 c.137G>A; MUTYH c.1187G>A were detected in Amsterdam I families and cosegregate with cancer. Analysis of OGG1 c.137G>A transcripts showed an inactivation of the splicing donor of exon 1.

**Conclusions:** Two rare variants (OGG1 c.137G>A; MUTYH c.1187G>A) and one common polymorphism (NUDT1 c.426C>T) were associated with CRC risk. We show that the BER pathway can play a significant role in a number of MSS-HNPCC colorectal cancers. More studies could be of interest in order to gain further understanding of yet unexplained CRC susceptibility cases. *Clin Cancer Res; 17(7); 1701–12. ©2011 AACR.*

**Introduction**

Hereditary nonpolyposis colorectal cancer (HNPPC) has been defined as a familial syndrome with an increased incidence of colorectal cancer and/or other extracolonic tumors (1, 2). Amsterdam I (3) and II (4) criteria were initially developed to describe common clinical features of HNPPC families. Approximately half of HNPPC cases are caused by defects in the DNA mismatch repair pathway (MMR; 5, 6). Germline mutations in mismatch repair genes (MLH1, MSH2, MSH6, and PMS2) are responsible for these cases and they are commonly referred to as Lynch syndrome. The other half of the Amsterdam families do not have any evidence of MMR deficiency and therefore their tumors are microsatellite stable (MSS). These MSS-HNPPC cases represent over 50% of all families fulfilling Amsterdam criteria representing a sizable number of cases presenting to the genetic counseling units. Three studies published almost simultaneously established this group as distinct from Lynch syndrome with a lower risk of colorectal cancer (CRC) and an older average age at diagnosis, though still much younger than sporadic cases (7–9). Lindor and colleagues (7) even proposed the term “familial colorectal cancer type X” (CRC-X), which is generally felt to be nondescript or potentially misleading (i.e., it implies that a genetic cause is located on the X chromosome). For this reason we think it is better to call them as MSS-HNPPC families.

The carcinogenic pathways involved in the development of CRC in MSS-HNPPC families are poorly understood. Several molecular features have been studied in...
The base excision repair (BER) pathway is the major pathway for oxidative DNA damage repair (13). In cancer, the most important damage caused by reactive oxygen species (ROS) is the oxidation of guanine, adenine, and thymine. The most stable product is 8-hydroxyguanine (8-OH-G) generated by the oxidation of guanine (14). 8-OH-G is highly mutagenic because it mispairs with cytosine and adenine with an almost equal efficiency during DNA replication. This leads to an increased frequency of G:C to T:A transversions that in oncogenes or tumor suppressor genes can lead to carcinogenesis (14, 15). The BER pathway identifies and repairs 8-OH-G incorporated into nascent DNA and it removes modified nucleosides from the pool. The main BER components are 8-oxoguanine DNA glycosylase (OGG1 [MIM 601982]) and mutY homolog (Escherichia coli; MUTYH [MIM 604933]). OGG1 is involved in the direct repair of 8-oxoguanine DNA glycosylase (16) and MUTYH is involved in the repair of A:8-OH-G mismatches due to its adenine glycosylase activity (17). The nucleoside diphosphate linked moiety X-type motif 1 (NUDT1 [MIM 600312]) also known as MTH1 acts indirectly on the BER pathway and hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP (18; Supplementary Fig. S1).

A deficiency in the BER pathway facilitates transversion mutations and therefore cancer risk. In fact, OGG1 has been mapped to the 3p26.2 region which has been seen to show loss of heterozygosity (LOH) in many cancers (19) and a high rate of transversions and 8-OH-G levels have been described in MSS and chromosome stable cell lines (20, 21). However, by far, the most striking finding so far has been the identification of bi-allelic mutations in the MUTYH gene as causing a significant number of attenuated familial adenomatous polyposis (AFAP) cases (19).

Regarding the potential role of variants of the BER genes as risk modifiers, some studies have described a possible role in different types of cancer (22–32). The results concerning risk of CRC or adenoma (CRA) have not always coincided (33–38).

In view of these results, we hypothesized that some MSS-HNPCC cases could be explained by some variants of the BER genes and an alteration of the BER pathway could underlie the carcinogenic process in some of these families.

Subjects and Methods

Study population

The study included colorectal cancer patients from 42 families. Thirty-six families were recruited from the Familial Cancer Clinic at the Hospital Clínico San Carlos (Madrid, Spain), and 6 from the Familial Gastrointestinal Cancer Unit of the University of Illinois at Chicago. All these families had patients with MSS tumors, normal expression of MMR proteins, and fulfilled the following criteria: (i) Amsterdam I/II (3, 4): 30 families; (ii) high-risk criteria (HRC): 12 families. We considered HRC families fulfilling all Amsterdam I/II criteria except for: (i) the earliest age of diagnosis was allowed to be up to 55 years, (ii) gastric cancer is included in HNPCC, or (iii) families with CRC under 50 in two or three generations but no first degree relatives affected. Affected family members with the earliest age at diagnosis were selected as probands. The main age of diagnosis in probands was 50 and the 54.5% were females.

This study was approved by the Institutional Review Boards of the Hospital Clínico San Carlos and the University of Illinois at Chicago. Informed consent was obtained from each participant (cases and controls). Personal and cancer family history was obtained from the proband and participating relatives. Cancer diagnoses and deaths were confirmed by reviewing medical records, pathology reports, or death certificates.

Control population

Controls were recruited from Hospital Clínico San Carlos and included 248 cancer-free persons with no family history. Ethnic backgrounds were comparable to the cases and all were from Spain. The mean age of controls was 51 and 58% were females.

DNA and RNA extraction

Peripheral-blood genomic DNA (gDNA) extraction was performed according to the salting out procedure (39) or with MagnaPure Compact extractor (Roche) according to the manufacturer’s recommended protocol.
Tumor gDNA was obtained from paraffin embedded tissues with a tumor content of more than 80% as determined by an experienced pathologist from hematoxylin/eosin-stained sections. Extractions were performed after digestion with proteinase K and purification with phenol/chloroform as previously described.

Peripheral-blood RNA extraction was performed with MagnaPure Compact extractor (Roche) according to the manufacturer's recommended protocol. SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) was used to synthesize cDNA using random hexamers according to the manufacturer instructions. DNA and RNA quantity and quality were assessed with Nanodrop (ND1000).

Microsatellite instability status

Microsatellite instability (MSI) analysis was performed testing the Bethesda panel of five microsatellite markers (BAT25, BAT26, D2S123, D5S346, and D17S250; 41) or the mononucleotide panel (41, 42) in paired tumor-normal DNA samples. Amplification and visualization of microsatellites were achieved as described previously (41, 42). Tumors were classified as MSS if all markers were stable.

Immunohistochemistry of Mlh1, Msh2, and Msh6

Immunohistochemistry (IHC) analysis of Mlh1, Msh2, and Msh6 proteins was done as described previously (41, 42). The percentage of positive nuclei was evaluated by two pathologists. Cases with >10% of nuclei staining were considered as positive protein expression.

Mutational screening of MLH1, MSH2, and MSH6

Mutation screening included the analysis of all coding sequences and intron/exon boundaries of MLH1, MSH2, and MSH6 by denaturing gradient gel electrophoresis as previously reported (43–45). In addition, the presence of genomic rearrangements at the MLH1, MSH2, MSH6, and PMS2 loci was tested by multiplex ligation-dependent probe amplification with P003 and P008 MRC-Kit, according to the supplier's instructions (MRC-Holland).

Mutational screening of OGG1, NUDT1, and MUTYH

Coding sequences and intron/exon boundaries of OGG1, NUDT1, and MUTYH genes were amplified and screened for mutations by direct sequencing. Primers for PCR amplification and sequencing were designed based on the sequences (Supplementary Table S1) and using the free online Primer3 v.0.4.0 program.

PCR reactions were performed in a total volume of 25 μL, comprising 50 ng of genomic DNA, 1 × PCR buffer (Ecogen SRL), 0.2 μM of each primer, 100 μM of each deoxynucleotide (Promega), MgCl2 at 1.5 mmol/L, 0.5 U Eco Taq DNA polymerase (Ecogen SRL). Reactions were performed in a thermal cycler under the following conditions: initial denaturation at 94°C for 4 minutes; amplification by 5 initial cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 30 seconds; and a final step of extension at 72°C for 7 minutes.

Amplified products were visualized in 2% agarose gels and nucleotide sequence was determined using Dye Terminator v1.1 Cycle Sequencing kit and ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The same oligonucleotides were used for both amplification and sequencing.

Segregation studies

In order to establish an association between the presence of the variant and the presence of disease, variants were screened in every available family member by direct sequencing. Primer pairs used in the mutational screening were also used for segregation studies.

In silico studies

The ClustalW2 alignment tool was used to check the level of conservation of the affected aminoacid in every missense variant. Polyphen and SIFT tools were used to predict the possible impact of every aminoacid substitution on the structure and function of the protein.

Human splicing finder (HSF) was used to study the potential effect of every variant on splicing.

Splicing studies

Transcript amplification. Pairs of primers located in different regions of OGG1 (Supplementary Table S2) were used to amplify cDNA as follows: PCR reactions were performed in a total volume of 20 μL, including 1 μL of cDNA product, 0.2 μM of each primer, dNTPs at 0.2 mmol/L each, MgCl2 at 2.5 mmol/L, 0.8 U AmpliTaq Gold DNA polymerase and 1× reaction buffer provided by the supplier (Applied Biosystems). Reactions were performed in a thermal cycler under the following conditions: initial denaturation at 95°C for 10 minutes; amplification with 35 cycles with denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 2 minutes (1 minute in the case of primer pair 1); and a final step of extension at 72°C for 7 minutes.

Amplified products were visualized in 2% agarose gels first and then in an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems), using GeneScan-500 LIZ (Applied Biosystems) as the size standard, according to the manufacturer's instructions. Nucleotide sequence was determined using Dye Terminator v1.1 Cycle Sequencing kit and model ABI PRISM 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. The same oligonucleotides were used for both amplification and sequencing.

Transcripts restriction analysis

PCR products of transcript amplification were digested with 5 U BsaWI, 1× Bsa (bovine serum albumin) and 1× NEBuffer 4 (New England BioLabs) at 60°C overnight and visualized using GeneScan-500 LIZ (Applied Biosystems) as size standard in an ABI PRISM 3130 Genetic Analyzer.
(Applied Biosystems) according to the manufacturer's instructions.

**Statistical analysis**

Distribution of genotypes in controls was tested for a departure from Hardy–Weinberg equilibrium by means of the χ² test. A P value of 0.05 was considered statistically significant.

χ² or Fisher's exact test was used to assess differences in allelic and genotype distribution between cases and controls. Specific risks were estimated by means of the OR with associated 95% CI by unconditional logistic regression. A test χ for trend (P<sup>trend</sup>) was used to evaluate difference in the risk by having more than one putative high-risk allele in those variants showing statistically significant differences in case–control study. Kaplan–Meier curves and Cox proportional hazard were used to analyze the disease free survival curves in the different genotypes of those variants showing statistically significant differences in case–control study. All tests were performed with the statistical software SPSS 13.0. Statistical power was estimated with EpInfo v.6.

**Results**

### Screening for variants in **OGG1**, **MUTYH**, and **NUDT1**

Sequencing results of the 3 main BER genes in probands of MSS-HNPCC families is shown in Table 1. Only two probands showed a wild type (WT) genotype in all studied genes and both were from Amsterdam I families. The rest showed **OGG1**, **NUDT1**, and/or **MUTYH** alterations in different combinations. Variant descriptions and frequencies are summarized in Table 2. Eight rare variants and 5 frequent variants were found in MSS-HNPCC patients. All variants were previously described by other authors except variant c.285C>T (A95A) in **OGG1**.

### Analysis of rare variants

Those variants showing a frequency lower than 5% were considered as "rare variants". We found 8 rare variants in a total of 8 probands out of 42. Segregation studies were done and in silico programs were used to estimate the level of amino acid conservation, protein damage prediction from amino acid substitutions, and possible splicing alterations (Table 2). Three variants: c.137G>A (R46Q) and c.923G>A (G308E) in **OGG1**, and the known variant c.1187T>A (known as G382D) in **MUTYH**, result in an amino acid substitution in highly conserved positions, and both prediction tools (Polyphen and SIFT) estimate a high damage in the functionality and/or structure of the protein. R46Q variant (24) was located in a highly conserved amino acid in the last nucleotide of **OGG1** exon 1 and causes the substitution of a basic amino acid for an acidic amino acid. This mutation was detected in family CC-298 (Supplementary Fig. S2). This was a family classified as HRC: four affected members' DNA.

**G382D variant** (19) was located in a highly conserved amino acid in **MUTYH** exon 13 and causes the substitution of an amino acid with an aliphatic group for one with an acidic group. This variant was detected in family CC-19 (Supplementary Fig. S2). The family fulfilled Amsterdam I criteria, and the variant was found in the three studied affected members. G308E variant (23) was located in a highly conserved amino acid in **OGG1** exon 6 and causes the substitution of an amino acid with an aliphatic group for one with an acidic amino acid. It was detected in family CC-CH6 (Supplementary Fig. S2). This was a family fulfilling Amsterdam I criteria. However, segregation studies were not possible because of unavailability of family members’ DNA.

Finally, c.285C>T (A95A) on **OGG1** had not been previously described. This new variant is a silent mutation, it does not cosegregate with cancer in the family, and it does not affect the splicing by in silico studies.

### Analysis of **OGG1-R46Q** transcripts

Only **OGG1-R46Q** showed a possible splicing alteration by the HSF prediction program. Previous studies have confirmed this prediction in lung and kidney tumor DNA that was homozygous for the mutation (24, 25). Both studies describe the inactivation of the c.137 splicing donor giving rise to transcripts containing the full length of exon 1 (1<sup>ox</sup>L1). Therefore, we decided to check this splicing alteration in germline DNA of our carrier patients. cDNA from the blood of a carrier was synthesized and two amplifications with different primer pairs (Supplementary Table S2) were carried out. Primer pair 1 (forward primer Ex1 located in exon 1 and reverse primer Ex3 located in exon 3) were designed to allow the amplification of 1<sup>ox</sup>WT transcript Ex1–Ex3 (Supplementary Fig. S3a) while primer pair 2 (forward primer Ex1 located in exon 1 and reverse primer In1a located in intron 1) were designed to selectively amplify 1<sup>ox</sup>L1 transcripts (Supplementary Fig. S3b). When primer pair 1 was used, 1<sup>ox</sup>WT transcripts were detected in carrier and control samples but no 1<sup>ox</sup>L1 transcripts were detected in any one.

In order to check if 1<sup>ox</sup>L1 transcript was actually expressed in our carrier samples we performed an amplification with primer pair 2. As shown in Figure 1B, cDNA of carrier samples expressed 1<sup>ox</sup>L1 transcripts whereas cDNA of control samples did not (data not shown).

The 46Q carriers have c.137G and c.137A alleles. c.137G WT sequence has a restriction target for BsaWI enzyme that is lost in the c.137A allele. Therefore, we can use BsaWI to distinguish between c.137G and c.137A alleles. The transcripts 1<sup>ox</sup>WT Ex1–Ex3 and 1<sup>ox</sup>L1 Ex1–In1 from both carrier and control samples were subjected to restriction analysis with BsaWI to check for allele origin (c.137G or c.137A).

1<sup>ox</sup>WT transcripts from noncarrier and carrier are shown in Figure 1A: c.137A allele was not detected in carrier cDNA so all 1<sup>ox</sup>WT transcripts come from the c.137G allele. 1<sup>ox</sup>L1 transcripts of carrier subject came exclusively from c.137A allele as it was expected (Fig. 1B). All transcripts were

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Table 1. Screening of OGG1, NUDT1, and MUTYH genes in probands of HNPCC-MSS families

| ID  | CRITERIA | MMR status | Dx. age | HIC | MSI | IHC | Mutation | Ber genes screening |
|-----|----------|------------|---------|-----|-----|-----|----------|---------------------|
| 275 | 42 AMS I  | NO(m)      | 53      | NO  | NO  | POS | WT       | WT                  |
| 334 | 49 AMS I  | NO(m)      | 68      | NO  | NO  | POS | WT       | WT                  |
| 377 | 80 AMS II | NO(m)      | 68      | NO  | NO  | POS | WT       | WT                  |
| 337 | 80 AMS II | NO(m)      | 68      | NO  | NO  | POS | WT       | WT                  |
| 492 | 53 AMS I  | NO(m)      | 53      | NO  | NO  | POS | WT       | WT                  |
| 498 | 47 AMS I  | NO(m)      | 53      | NO  | NO  | POS | WT       | WT                  |
| 510 | 48 AMS I  | NO(m)      | 48      | NO  | NO  | POS | WT       | WT                  |
| 519 | 48 AMS I  | NO(m)      | 48      | NO  | NO  | POS | WT       | WT                  |
| 692 | 47 AMS I  | NO(m)      | 47      | NO  | NO  | POS | WT       | WT                  |
| 973 | 47 AMS I  | NO(m)      | 47      | NO  | NO  | POS | WT       | WT                  |
| 1218| 42 AMS I  | NO(m)      | 42      | NO  | NO  | POS | WT       | WT                  |
| 1222| 52 AMS I  | NO(m)      | 52      | NO  | NO  | POS | WT       | WT                  |
| 1225| 54 AMS I  | NO(m)      | 54      | NO  | NO  | POS | WT       | WT                  |

(Continued on the following page)
| ID  | CRITERIA | Dx | age | MSI     | IHC | Mutation                  | OGG1                      | NUDT1 | MUTYH             |
|-----|----------|----|-----|---------|-----|---------------------------|---------------------------|-------|------------------|
| 1860| AMS I    | 57 | NO  | WT      |     | c.137G>A (p.Arg46Gln)     | WT                        |       | c.504+35G>A      |
| 1921| AMS I    | 46 | NO  | WT      |     | c.74815>C/G.c.977G>G     | WT                        |       | c.1014G>C (p.Gln338His) |
| 1901| AMS II   | 45 | NO  | WT      |     | WT                        | c.426C>T(p.Asp142Asp)     |       | c.504+35G>A      |
| 2042| AMS I    | 47 | NO  | WT      |     | c.74815>C/G.c.977G>G     | c.426C>T(p.Asp142Asp)     |       | c.690+21C>A       |
| 2174| AMS I    | 24 | NO  | WT      |     | c.74815>C/G.c.977G>G     | WT                        |       |                  |
| 2235| AMS I    | 54 | NO  | POS     |     | c.74815>C/G.c.977G>G     | WT                        |       | c.1014G>C (p.Gln338His)' |
| 2346| AMS I    | 72 | NO  | POS     |     | c.74815>C/G.c.977G>G     | c.426C>T(p.Asp142Asp)     |       | c.1014G>C (p.Gln338His) |
| 2310| HR       | 51 | NO  | WT      |     | c.74815>C/G.c.977G>G     | WT                        |       | c.1014G>C (p.Gln338His) |
| 2347| AMS I    | 42 | NO  | POS     |     | c.74815>C/G.c.977G>G     | c.426C>T(p.Asp142Asp)     |       | WT               |
| 2496| AMS I    | 40 | NO  | POS     |     | c.74815>C/G.c.977G>G     | c.426C>T(p.Asp142Asp)     |       | WT               |
| 2695| HR       | 51 | NO  | POS     |     | c.74815>C/G.c.977G>G     | c.426C>T(p.Asp142Asp)     |       | WT               |
| 1252| HR       | 49 | NO  | WT      |     | c.74815>C/G.c.977G>G     | c.426C>T(p.Asp142Asp)     |       | c.504+35G>A      |
| 1524| AMS I    | 40 | NO  | WT      |     | c.74815>C/G.c.977G>G     | c.426C>T(p.Asp142Asp)     |       | WT               |
| 1639| AMS I    | 43 | NO  | WT      |     | c.74815>C/G.c.977G>G     | 5.36C>T(p.Asp122Asp)/     |       | WT               |
| 5653| AMS I    | 54 | NO  | WT      |     | c.74815>C/G.c.977G>G     | c.426C>T(p.Asp142Asp)     |       | WT               |
| 7097| AMS II   | 43 | NO  | WT      |     | c.74815>C/G.c.977G>G     | c.426C>T(p.Asp142Asp)     |       | WT               |
| 11074|AMS I   | 67 | NO  | WT      |     | c.923G>A (p.Try308Glu)   | c.426C>T(p.Asp142Asp)     |       | c.1014G>C (p.Gln338His) |

CRITERIA: Family criteria.
AMS I: Amsterdam I criteria; AMS II: Amsterdam II criteria; HR: families fulfilling all Amsterdam I/II criteria with some exceptions (i) the earliest age of diagnosis up to 55 years, (ii) gastric cancer is included in HNPCC, and (iii) families with a high number of CRC affected subjects under 50 in two or three generations but no first degree relatives affected. POS: positive.
### Table 2. OGG1, NUDT1, and MUTYH variants found in the MSS-HNPCC population

| Variant | HGVS | Reference | Allele frequency | Segregation | In silico tests | CLUSTALW2 | POLYPHEN² (PSIC) | SIFT | HSF³ (CV) |
|---------|------|-----------|-----------------|-------------|----------------|-----------|------------------|------|-----------|
| OGG1    | c.137G>A | Kohno 1998 | 1.19 | Yes | HIGH | PD | 0.2327 | DEL | No |
|         | c.285C>T | (p.Arg46Gln) | 1.19 | No | No | na | na | na | DEL |
| NUDT1   | V106M | Wu 1995 | 1.19 | No | VERT | B(1.43) | DEL | No |
| MUTYH   | G386D (G382D) | Al-Tassan 2002 | 1.25 | Yes | HIGH | PD | 0.242 | DEL | No |
|         | IVS4/C0 15 | Kohno 1998 | 22.62 | No | No | na | na | na | No |
|         | G308E | Blons 1999 | 1.19 | No | No | na | na | na | No |
|         | S326C | Kohno 1998 | 21.43 | No | No | na | na | na | No |
|         | T122D | Wu 1995 | 34.52 | No | No | na | na | na | No |
|         | IVS8/C0 21 | Isidro 2004 | 1.25 | No | No | na | na | na | No |
|         | IVS12/C0 27 | Peterlongo 2006 | 1.25 | No | No | na | na | na | No |
|         | S338H (S324H) | Slupska 1996 | 21.25 | No | No | B(1.409) | TOL | No |
|         | G396D (G382D) | Al-Tassan 2002 | 1.25 | Yes | HIGH | PD | 0.2142 | DEL | No |

- **Alignment program CLUSTALW2**: HIGH: conserved from vertebrates to bacteria; LOW: not conserved even in vertebrates; VERT: conserved only in vertebrates.
- **Protein damage prediction program POLYPHEN²**: PD: probably damaging; B: benign.
- **Protein damage prediction program SIFT**: DEL: deleterious effect; TOL: tolerant.
- **Splicing alteration prediction program HSF**: CV: consensus values.
- **ni**: not informative.
- **na**: not applicable.

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confirmed by sequencing and the insertion of intron 1 (521bp) could be detected in mutant transcripts but not in controls as expected because 1αL1 transcript could not be amplified in controls (data not shown).

Association studies with frequent variants

Variants showing a frequency higher than 10% were considered as “frequent variants” (Table 2). In order to determine a potential association between each variant and colorectal cancer risk, association case–control studies were made. One randomly selected affected member of each family was included in the case group and it was compared with the control population. Case–control studies were performed in all frequent variants but not in c.748C>C015G (IVS4>C0115) in OGG1 because it showed a strong linkage with c.977C>G (S326C) in OGG1 variant (see Table 1). As shown in Table 3, only c.426C>T (D142D) variant in NUDT1 showed a strong association with our population (OR = 2.23; 95% CI = 1.35–3.66; ρ = 0.003). Distribution of alleles of all frequent variants in both cases and controls were in Hardy–Weinberg equilibrium (data not shown).

**NUDT1-D142D analysis**

In order to inquire about the effect of this variant on the study population, we conducted genotype frequency comparisons between cases and controls to check for allelic dose effect. Kaplan–Meier curves and Cox proportional hazard were used for disease-free survival analysis. As shown on Table 4, the OR increased significantly with one allelic dose (OR = 2.61; ρ = 0.009) and subjects with two allelic doses showed an even higher OR (OR = 3.66; ρ = 0.035). Chi for trend test showed a significant value (ρ = 0.007) for the increase of the CRC risk according to the allelic dose (Table 4). Figure 2 shows disease-free survival curves for the different genotypes of the NUDT1-D142D variant. As shown, homozygous subjects for the mutant allele show a decrease in the age of onset of HNPCC tumors (HR = 2.55; 95% CI= 1.06–6.13; ρ = 0.036) whereas

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**Table 3. Comparison of allelic frequencies between cases and controls in OGG1, NUDT1, and MUTYH variants**

| Variant  | M allele cases (%) | M allele controls (%) | OR (CI = 95%) | ρ    |
|----------|--------------------|----------------------|---------------|------|
| OGG1     |                    |                      |               |      |
| IVS4−15  | 14/84 (16.7)       | 104/516 (20.2)       |               |      |
| OGG1     | S326C              |                      |               |      |
| 30/84 (35.7) | 99/496 (19.9)       | 2.23 (1.35–3.66)    | 0.003         |
| NUDT1    | D142D              |                      |               |      |
| IVS6−35  | 12/76 (15.8)       | 23/164 (14)          |               |      |
| MUTYH    | Q338H (Q324H)      |                      |               |      |
| 15/78 (19.2) | 31/168 (18.4)       | ns*                 |               |      |

*ns: not significant.

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heterozygous have no different age of onset from the WT group. The mean age of onset was 60 years in WT and heterozygous whereas it was 51 years in homozygous.

Discussion

We have screened for variants in the three main genes of BER pathway OGG1, MUTYH, and NUDT1 in 42 MSS HNPCC probands, and we have found variants in some of these genes in 40 probands (95%).

Rare variants

Among these variants, only OGG1-A95A had not been previously described and, according to our findings, it does not increase CRC risk. Three missense variants (MUTYH-G382D, OGG1-G308E, and OGG1-R46Q) affected highly conserved amino acids (from mammals to bacteria) and a highly damaging effect was predicted. The MUTYH-G382D variant was present in all tested affected members in the corresponding family. This variant when homozygosis or in combination with another MUTYH mutation is known to be responsible for some familial attenuated polyposis cases (19, 46, 47). Some studies have also shown an increased risk of CRC in patients with monoallelic MUTYH mutations (35, 37, 48). Moreover, it has been suggested that some MSS-HNPCC families are enriched with monoallelic MUTYH mutations (11). Therefore, we cannot rule out the possible role of this variant as CRC risk modifier in the described family.

The OGG1-G308E variant was previously described in oral (23) and renal (25) cancer. Unfortunately, we could not perform segregation analysis but this is an unlikely pathogenic variant (23). Variant OGG1-R46Q was described as homozygous in a lung tumor cell line (24) and a renal tumor (25), and as heterozygous in the germ-line of a lung cancer patient (49). This is potentially deleterious for the protein’s functionality and therefore it may increase CRC risk. Thus, assays have shown a lower

| Table 4. Allele dose effect of NUDT1-D142D variant on the CRC risk in MSS-HNPCC |
|-----------------------------|------------------|------------------|------------------|------------------|
| Genotype frequency comparisons to assess dose effect on OR |
| Dose allele | Carriers in cases | Carriers in controls | OR (CI = 95%) | p |
|-----------------|------------------|------------------|------------------|------------------|
| CT/TT vs. CC | Any | 25/42 | 86/248 | 2.77 (1.42–5.41) | 0.003 |
| CT vs. CC | Single | 20/37 | 73/235 | 2.61 (1.29–5.27) | 0.009 |
| TT vs. CC | Double | May-22 | 13/175 | 3.66 (1.16–11.53) | 0.035 |
| Chi for trend to assess significance of the difference on dose effect |
| Genotype | Carriers in cases | Carriers in controls | p |
|-----------------|------------------|------------------|------------------|
| CC | 17/42 | 20/42 | May-42 | 0.007 |
| CTRLs | 162/248 | 73/248 | 13/248 |

CTRLs: controls.

Figure 2. Analysis of disease-free survival according to NUDT1-D142D genotypes. A, three possible genotypes are compared. CC: wild type genotype. Mean onset age: CC = 60.1 ± 3.7; CT = 60.6 ± 4; TT = 51.3 ± 5.3. B, WT genotypes plus heterozygous are compared together against recessive genotype TT. Mean onset age: CC/CT = 60.5 ± 2.7; TT = 51.3 ± 5.3. The curves suggest that NUDT1-D142D variant acts as a recessive risk modifier decreasing the cancer’s age of onset in homozygous subjects from MSS-HNPCC families.
activity of the variant protein in comparison with the WT protein (24, 25, 50, 51). Moreover, the point mutation is located in the last nucleotide of exon 1 corresponding to a splicing region. Previous studies confirmed the inactivation of the splicing donor giving rise to transcripts containing the full length of intron 1 and leading to a premature stop codon just after exon 1 (24, 25). Both studies analyzed the transcript analysis in homozygous tumor samples and they detected both, WT and mutant (II) transcripts. Audebert and colleagues explained the presence of the WT transcript as DNA contamination (25). We confirmed the mutant transcript in the germline DNA of our heterozygous carrier patients. However, we could only detect the presence of II transcript with the use of specific primers, which made us think that either the expression of II transcript is very low, in comparison with WT transcript, or II transcript is partially degraded. Moreover, through restriction analysis we saw that all WT transcripts detected came from the WT allele. So we hypothesize that c.137A mutant allele has heavily inactivated the splicing donor of exon 1 and the II transcript tends to degrade. Therefore, in heterozygous condition probably the only functional transcript comes from the c.137G allele and the contribution of the c.137A allele is negligible. Probably decreased levels of WT transcript are affecting the correct OGG1 functionality. Segregation analysis showed that the variant was present in all affected family members. We could not confirm LOH as no tumor DNA was available.

Frequent variants

Five frequent variants were found, IVS-15 and S326C in OGG1, D142D in NUDT1, and 2 in c.504+35G>A (IVS6+35) and c.1014G>C (Q324H) MUTYH. Case–control studies were performed for all except IVS-15 because this was closely linked to the variant S326C and linkage had been previously observed (24).

Only variant D142D in NUDT1 showed a significant association with CRC risk in MSS-HNPCC patients when mutant allelic frequency was compared between cases and controls (OR = 2.23; 95% CI = 1.35–3.66; p = 0.003), and also when genotypes were compared (OR = 2.77; 95% CI = 1.42–5.41; p = 0.003). In order to figure out if the effect of this variant was dominant or recessive, we assessed the effect of allele dosage by comparing the different NUDT1-D142D genotypes in cases and controls. We observed an incremental risk according to the increase in allele dose (p = 0.009). Then, we studied the effect of this variant in the age of onset of cancer. Homozygous showed a decrease of 9 years in the mean age of onset (HR = 2.55; 95% CI = 1.06–6.13; p = 0.036), while no effect was seen in heterozygous. Therefore, variant NUDT1-D142D acts as a risk modifier in our MSS-HNPCC patients and it increases CRC risk according to allele dose. As this variant is silent, and no prediction of splicing alteration was found by the HSF program, it is reasonable to think that NUDT1-D142D is probably linked to a real low penetrance allele and it does not cause the increased CRC risk by itself.

The NUDT1-D142D has been described for the first time in germline of patients with HNPPC by Wu and colleagues (52). In the other hand, Görgens and colleagues (31) analyzed this variant in 29 squamous cell carcinomas of the head and neck (SCCHN) cases and 30 controls and they did not find an association between NUDT1-D142D and SCCHN. It is interesting to remark that the minor allele frequency (MAF) in our control group was similar to Görgens and colleagues (31) control group (19.9% vs. 16.7%). Therefore, our work is the first evidence of association between this silent variant of NUDT1 and CRC risk. Further studies in larger populations should be done to confirm the role of NUDT1-D142D in cancer risk.

The rest of the variants did not show any difference between cases and controls. Variant OGG1-S326C has been associated with a decreased ability to suppress mutagenesis. Furthermore, some studies have suggested an association of this variant with different cancer types such as lung (27), mouth (26), kidney (32), and prostate (28). Results have been inconsistent for an association with colorectal cancer (34, 36, 38, 53) and two studies found an association only in smokers (33–37). No significant association was found with our series of MSS-HNPCC patients. Finally, no association was found with MUTYH variants Q324H and IVS6-35.

An important limitation of our study is that we were only able to detect OR of 2.2 with a power of 80% and a CI of 95% due to sample size. Therefore, we cannot rule out the possibility of a lower effect of any of these variants, which could not be detected with our sample size. The analysis in larger cohorts of MSS-HNPCC families could help clarify this issue.

In spite of this limitation, we show that the two rare variants (OGG1 c.137G>A; MUTYH c.1187G>A) and one common polymorphism (NUDT1 c.426C>T) were associated with CRC risk in a limited number of MSS-HNPCC families.

Supplemental data

Supplemental data include three figures describing ROS effect on DNA sequence and action points of the main BER pathway genes, pedigrees of families carrying rare BER variants, and splicing analysis of OGG1-R46Q variant and two tables showing the oligonucleotide sequences used in this work.

Web resources

The following online computer programmes were used in this work:

Primer3 v.0.4.0: http://fokker.wi.mit.edu/primer3/input.htm
ClustalW2: http://www.ebi.ac.uk/Tools/clustalw2/index.html
Polyphen: http://genetics.bwh.harvard.edu/pph/index.html
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis of the Oxidative Damage Repair Genes NUDT1, OGG1, and MUTYH in Patients from Mismatch Repair Proficient HNPCC Families (MSS-HNPCC)

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