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Running Title: *MUTYH* mutations and breast and colorectal cancer families

Preclinical study

Increased *MUTYH* mutation frequency among Dutch families with breast cancer and colorectal cancer

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Abstract

Homozygous and compound heterozygous *MUTYH* mutations predispose for *MUTYH*-associated polyposis (MAP). The clinical phenotype of MAP is characterized by the multiple colorectal adenomas and colorectal carcinoma. We previously found that female MAP patients may also have an increased risk for breast cancer. Yet, the involvement of *MUTYH* mutations in families with both breast cancer and colorectal cancer is unclear. Here, we have genotyped the *MUTYH* p.Tyr179Cys, p.Gly396Asp and p.Pro405Leu founder mutations in 153 Dutch families with breast cancer patients and colorectal cancer patients. Families were classified as polyposis, revised Amsterdam criteria positive (FCRC-AMS positive), revised Amsterdam criteria negative (FCRC-AMS negative), hereditary breast and colorectal cancer (HBC) and non-HBC breast cancer families. As anticipated, biallelic *MUTYH* mutations were identified among 13% of 15 polyposis families, which was significantly increased compared to the absence of biallelic *MUTYH* mutations in the population ($P=0.0001$). Importantly, six heterozygous *MUTYH* mutations were identified among non-polyposis families with breast and colorectal cancer. These mutations were identified specifically in FCRC-AMS negative and in HBC breast cancer families (11% of 28 families and 4% of 74 families, respectively; $P=0.02$ for both groups combined vs. controls). Importantly, the 11% *MUTYH* frequency among FCRC-AMS negative families was almost 5-fold higher than the reported frequencies for FCRC-AMS negative families unselected for the presence of breast cancer patients ($P=0.03$). Together, our results indicate that heterozygous *MUTYH* mutations are associated with families that include both breast cancer patients and colorectal cancer patients, independent of which tumor type is more prevalent in the family.

Key words: Familial breast cancer; Familial colorectal cancer; Genetic predisposition; HBC; *MUTYH*

Introduction

Germline mutations in the base excision repair gene *MUTYH* are associated with the recessively inherited colorectal cancer syndrome *MUTYH*-associated polyposis (MAP) that is characterised by multiple colorectal adenomas and colorectal carcinoma [1-4]. Homozygous and compound heterozygous *MUTYH* mutations have been identified in about 25% of patients with polyposis [5, 6]. Two prevalent *MUTYH* mutations, c.536A>G, p.Tyr179Cys and c.1187G>A, p.Gly396Asp (previously annotated as c.494A>G, p.Tyr165Cys and c.1145G>A, p.Gly382Asp, see patients and methods), account for approximately 75% of the reported mutations in MAP patients thus far [5]. In addition, several other *MUTYH* founder mutations have been identified, including the Dutch *MUTYH* c.1214C>T, p.Pro405Leu founder mutation (previously c.1172C>T, p.Pro391Leu) [6]. Together, the *MUTYH* p.Tyr179Cys, p.Gly396Asp and p.Pro405Leu mutations represent approximately 90% of *MUTYH* mutations reported in Dutch MAP patients [6, 7].

The clinical phenotype associated with MAP overlaps with the familial adenomatous polyposis (FAP) and attenuated familial adenomatous polyposis (AFAP) phenotypes [5, 8]. The number of colorectal adenomas in MAP patients ranges from five to a few hundreds, generally presenting during the 5th decade of life. About half of MAP patients presents with colorectal cancer at the time of diagnosis [5, 8, 9]. In addition, germline *MUTYH* mutations have been identified in colorectal cancer patients with a cancer phenotype more reminiscent to Lynch syndrome (also known as hereditary non-polyposis colorectal cancer) [10-16]. Recently, we have identified a relatively high prevalence of breast cancer (18%) among Dutch female biallelic *MUTYH* mutation carriers, suggesting that breast cancer may be part of the clinical phenotype associated with MAP [6]. However, a recent Canadian study did not detect an association between *MUTYH* mutations and an increased breast cancer risk [17]. Here we have evaluated whether *MUTYH* mutations predispose to breast cancer in the context of familial colorectal cancer, by screening the *MUTYH* p.Tyr179Cys, p.Gly396Asp and p.Pro405Leu founder mutations in 153 Dutch families with both breast cancer patients and colorectal cancer patients.

Patients and Methods

Cancer family and population control cohorts

The 153 families with both breast cancer and colorectal cancer included in this study were ascertained by two academic cancer centres in the South-Western Netherlands: Leiden University Medical Centre (LUMC) in Leiden and Erasmus University Medical Centre (Erasmus MC) in Rotterdam. The LUMC cohort included 37 cancer families selected from 565 non-*BRCA1/BRCA2* breast cancer families and 46 cancer families selected from 311 colorectal cancer families without a mismatch repair gene defect and/or a germline *APC* mutation, after molecular genetic screening according to Hendriks *et al.* [18]. The selected 83 LUMC cancer families each had at least one breast cancer patient and at least one colorectal cancer patient within second degree relatives (DGRs) and were registered by either the Clinical Genetic Centres of Leiden and Rotterdam, or the Dutch Foundation for Detection of Hereditary Tumors (STOET). The Erasmus MC cohort included 70 breast cancer families with hereditary breast and colorectal cancer (HBCC) as defined by Meijers-Heijboer *et al.* ([19], and below). Germline *BRCA1* and *BRCA2* mutations were identified in fourteen and three of these 70 HBCC breast cancer families, respectively. The Erasmus MC cohort was selected from 578 breast cancer families registered by the Rotterdam Family Cancer Clinic at Erasmus MC [19, 20].

The 153 selected breast and colorectal cancer families were classified in six categories (Table 1, Supplementary Table 1), including families with: 1, polyposis; 2, colorectal cancer and positive for the revised Amsterdam criteria (FCRC-AMS positive); 3, colorectal cancer and negative for the revised Amsterdam criteria (FCRC-AMS negative); 4, HBCC; 5, non-HBCC breast cancer; and 6, mixed families with breast and colorectal cancer that could not be assigned to any of these five categories. To assign each family to only a single category of families, the six categories were ranked with the highest rank assigned to category 1. Families were classified as polyposis families (n=15) when at least one patient in the family was diagnosed with many colorectal adenomas (more than 15, n=2) or when at least two patients in the family were diagnosed with an unknown number of colorectal

adenomas (n=13). Families were classified as FCRC-AMS positive (n=8) when they included at least three relatives with a Lynch syndrome-associated tumor (colorectal, small bowel, endometrial, ureter or renal pelvis) *and* one patient was a first DGR of the other two *and* two successive generations were affected *and* one patient was diagnosed before age 50 years (revised Amsterdam criteria, [21]). Families were classified as FCRC-AMS negative (n=28) when they did not fulfil the revised Amsterdam criteria but included at least two colorectal cancer patients in first DGRs of whom at least one was diagnosed before age 50 years (n=13) *or* at least three colorectal cancer patients of whom at least one is a first DGR of the other colorectal cancer patients (n=15). Families were classified as HBCC families (n=74) when they fulfilled the breast cancer family criterion and in addition included at least one patient with breast and colorectal cancer *or* at least one patient with colorectal cancer diagnosed before age 50 years who is within second DGR of a breast cancer patient *or* at least two colorectal cancer patients of whom at least one is within second DGR of a breast cancer patient [19]. Non-HBCC breast cancer families (n=13) included at least two first or second DGRs with breast cancer of whom at least one was diagnosed before age 60 years. Fifteen families could not be classified according to any of these criteria. On average these fifteen “mixed” families included two colorectal cancer patients and one breast cancer patient.

The 1192 population controls originated mainly from the South-Western Netherlands and included a hospital-based cohort of 165 spouses of members from breast cancer families, a hospital-based cohort of 254 individuals screened for non-cancer related genetic diseases, and a population-based cohort of 773 randomly-selected blood donors.

The Medical Ethical Review Boards of Leiden University Medical Centre and Erasmus University Medical Centre have approved this study and informed consent had been obtained from all patients.

MUTYH mutation analysis

MUTYH (ENSG00000132781) mutations were annotated according to the most up-to-date *MUTYH* annotation (www.lovd.nl/MUTYH and NM_001128425.1, <http://www.ncbi.nlm.nih.gov/nucore/190358496>). The two most common *MUTYH* founder

mutations p.Tyr179Cys and p.Gly396Asp as well as the Dutch *MUTYH* p.Pro405Leu founder mutation (previously known as p.Tyr165Cys, p.Gly382Asp and p.Pro391Leu, respectively) were screened in blood-derived DNA of the index patient from each cancer family. In the majority of families, the index patient was the breast cancer or colorectal cancer patient with the youngest age at diagnosis for whom DNA was available. *MUTYH* p.Tyr179Cys, p.Gly396Asp and p.Pro405Leu mutation screening included amplification of exons 7 (p.Tyr179Cys) and 13 (p.Gly396Asp and p.Pro405Leu) of the *MUTYH* gene by standard PCR as described.[6,7,22]. Amplified fragments were subsequently sequenced with BigDye™ Terminator v3.0 or v3.1 Ready Reaction Cycle mix (Applied Biosystems) and analyzed on an ABI-3100 or 3730 capillary sequencer. Analysis of the sequence data was performed using SeqScape software (Applied Biosystems) or Mutation Surveyor™ software (SoftGenetics, LLC). The *MUTYH* p.Tyr179Cys, p.Gly396Asp and p.Pro405Leu mutation frequency in the control cohorts was determined by three mutation-specific Taqman® assays designed by Assays-by-designSM Service (Applied Biosystems). Samples were run on an ABI-7500 Analyzer and allelic discrimination was performed using SDS software (Applied Biosystems). No differences were identified for 238 genotypes that had been screened by Taqman analysis and sequencing, indicating that the sensitivity for detection of *MUTYH* mutations is similar for both techniques. All mutant *MUTYH* p.Tyr179Cys, p.Gly396Asp and p.Pro405Leu samples were confirmed by analysis of an independently generated template. For the six heterozygous *MUTYH* mutation carriers, the entire *MUTYH* coding sequence including exon-intron boundaries has been analysed for additional genetic alterations. Primer sequences, PCR, Taqman and sequence conditions are available on request.

Statistical analysis

The difference between the *MUTYH* p.Tyr179Cys, p.Gly396Asp and p.Pro405Leu mutation frequency in the various cohorts of cancer patients versus controls was analysed using Fisher's Exact Test. Odds ratios (OR) and 95% confidence intervals (CI) were calculated from two-by-two tables. *P*-values of 0.05 or smaller were considered significant. All statistical

analyses were performed with STATA statistical package, release 10 (STATA Corp, College Station, TX).

Results and discussion

Biallelic *MUTYH* inactivation in polyposis families with breast and colorectal cancer

Genotyping of the *MUTYH* p.Tyr179Cys, p.Gly396Asp and p.Pro405Leu founder mutations in 153 families with breast and colorectal cancer identified eight *MUTYH* mutant families, including two families with compound heterozygous index patients and six families with heterozygous index patients (Table 1). In addition, we detected the pathogenic *MUTYH* mutation (c.1227_1228dup, p.Glu410GlyfsX43, previously c.1185_1186dup, p.Glu396GlyfsX43; one family) and two intronic polymorphisms (c.504+35G>A, previously c.462+35G>A; 29 families and c.1187-27C>T, previously c.1145-27C>T; three families) [23].

The two *MUTYH* families with compound heterozygous index patients were both polyposis families, with family 20090 carrying the *MUTYH* p.Tyr179Cys and p.Pro405Leu mutations and family 53119 carrying the *MUTYH* p.Tyr179Cys and p.Gly396Asp mutations (Table 1 and Table 2). The index patient from family 20090 was diagnosed with colorectal cancer at age 40 years. DNA of two first-degree affected family members was available for additional *MUTYH* genotyping, revealing that the mother of the index patient (diagnosed with colorectal cancer at age 81 years) was a heterozygous *MUTYH* p.Tyr179Cys carrier and a brother of the index patient (diagnosed with colorectal cancer and adenomas at age 50 years) was a compound heterozygous *MUTYH* p.Tyr179Cys and p.Pro405Leu carrier. The index patient from family 53119 was diagnosed with colorectal cancer and adenomas at age 46 years. DNA from one first-degree affected family member was available for additional *MUTYH* genotyping, revealing that a sister of the index patient (diagnosed with breast cancer at age 63 years) carried a heterozygous *MUTYH* p.Tyr179Cys mutation (Table 2). Together, 2 of 15 (13.3%) polyposis families carried compound heterozygous pathogenic *MUTYH* mutations as compared to none of 1192 population controls ($P=0.0001$; Table 1). Previous reports had identified homozygous and compound heterozygous *MUTYH* mutations in approximately 10%

of FAP patients and in 20-25% of AFAP patients [3, 6, 24]. Our results are in line with these reports and hence confirm the reported association of biallelic *MUTYH* mutations with a polyposis phenotype.

Heterozygous *MUTYH* mutations in non-polyposis families with breast and colorectal cancer

Heterozygous *MUTYH* mutations were identified in 3 of 28 (10.7%) FCRC-AMS negative families, including two families with index patients carrying the *MUTYH* p.Gly396Asp mutation and one family carrying *MUTYH* p.Pro405Leu (Table 1 and Table 2). Of these three FCRC-AMS negative families, family 50483 was classified as FCRC-AMS negative because it included two colorectal cancer patients in first DGRs of whom one was diagnosed before age 50 years, whereas families 10075 and 54058 included at least three colorectal cancer patients of whom at least one was a first DGR of the other colorectal cancer patients. DNA of non-index cases from two families (10075 and 50483) was available for additional genotyping. Yet, no *MUTYH* mutations were identified among the three breast cancer patients and the single patient with breast cancer and vulva melanoma (Table 2). The 10.7% heterozygous *MUTYH* mutation frequency among FCRC-AMS negative families was significantly increased compared to the 1.9% heterozygous *MUTYH* mutation frequency among controls (Table 1, $P=0.02$) and was also higher than the absence of mutations among the nine FCRC-AMS positive families. Thus far, seven studies have evaluated *MUTYH* in familial colorectal cancer patients who did not fulfil the revised Amsterdam and/or Bethesda criteria, together including 866 cases [10-16]. The heterozygous *MUTYH* mutation frequencies ranged from 0% to 5.7%, with an average *MUTYH* mutation frequency of 2.3% (20/866). Thus, the reported *MUTYH* mutation frequency among such colorectal cancer families is about 5-fold lower than we have observed among our FCRC-AMS negative families (2.3% versus 10.7%; $P=0.03$). In contrast to the earlier reported FCRC-AMS negative families, we have selected FCRC-AMS negative families that included at least one breast cancer patient. It seems plausible that the dissimilarity in *MUTYH* mutation frequency reflects this difference in selection criteria, suggesting that women with heterozygous *MUTYH* mutations from families that classify as FCRC-AMS negative are at increased risk for breast cancer.

The *MUTYH* p.Tyr179Cys, p.Gly396Asp and p.Pro405Leu mutations were each identified once among the 74 HBCC families (Table 1 and Table 2). Two of the *MUTYH* HBCC families (57466 and 59168) classified as HBCC families because they included a colorectal cancer patient diagnosed before age 50 years. HBCC family 15138 included two colorectal cancer patients of whom one was a first DGR of a breast cancer patient. DNA of non-index cases was available only for family 57466, revealing that a sister of the index patient (diagnosed with breast cancer at age 46 years) did not carry the *MUTYH* p.Tyr179Cys mutation (Table 2). Together, 3 of 74 (4.1%) HBCC families carried heterozygous *MUTYH* mutations, which was higher but not significantly different from the 1.9% population frequency (Table 1). However, it is important to realize that our classification method by ranking allowed assignment of families to only a single category. As a result, families that fulfilled the criteria for both FCRC-AMS negative and HBCC were classified as FCRC-AMS negative families. Indeed, 17 of 28 FCRC-AMS negative families (61%) also met the HBCC criteria, as also four families from other categories. Moreover, two of the 17 FCRC-AMS negative families carried *MUTYH* mutations. In total, 5 of 95 (5.3%) HBCC families were heterozygous *MUTYH* mutant which was increased compared to controls ($P=0.05$; Supplementary Table 1), suggesting that *MUTYH* mutations also associate with HBCC. To further evaluate the involvement of heterozygous *MUTYH* mutations with HBCC, we re-classified all non-polyposis families according to the number of breast cancer and colorectal cancer patients in the family, resulting in three categories with more, equal, or less colorectal cancer than breast cancer patients (Table 3). This analysis showed that the *MUTYH* mutation frequency is increased among families with more colorectal cancer than breast cancer patients (7.1%) as also among families with more breast cancer than colorectal cancer patients (5.8%), and that these frequencies were rather similar.

In conclusion, our analyses suggest that heterozygous *MUTYH* mutations are associated with a breast and colorectal cancer phenotype, even though there was incomplete cosegregation of *MUTYH* mutations with breast cancer (Table 2). Heterozygous *MUTYH* mutations were identified specifically in families that classified as FCRC-AMS negative and HBCC. Importantly, the families may present with more colorectal cancer patients than breast cancer patients or vice versa. Also, the incomplete segregation between heterozygous

MUTYH mutations with the cancer phenotype and the rather low cancer risks associated with these heterozygous *MUTYH* mutations (RR=2.0) may suggest an as yet unrecognized low-risk polygenic disease mechanism [25]. More research is required to confirm our results in larger series but particularly to more precisely define the clinical criteria for the *MUTYH*-associated breast and colorectal cancer phenotype.

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Classification of families with breast and colorectal cancer	<i>MUTYH</i> +/Total tested (%)	P-value (OR; 95% CI)
Controls	23/1192 (1.9)	
1. Polyposis families	2/15 ^a (13.3)	0.0001 [†] (92.7; 54.0-159.2)
2. FCRC-AMS positive families	0/8	n.s. [†]
3. FCRC-AMS negative families	3/28 (10.7)	0.02 [†] (5.5; 1.8-17.1)
I) 2x CRC 1 st DGR + 1 CRC _{≤50} y	1/13	
II) 3x CRC 1 st DGR + 1 st DGR others	2/15	
4. HBCC families	3/74 (4.1)	n.s. [†]
5. Non-HBCC breast cancer families	0/13	n.s. [†]
6. Other, mixed families	0/15	n.s. [†]

Legends to the tables

Table 1 Prevalence of *MUTYH* p.Tyr179Cys, p.Gly396Asp and p.Pro405Leu mutations among 153 Dutch families with breast cancer and colorectal cancer.

^{*}, P-value for cancer patients with compound heterozygous *MUTYH* mutations versus controls with compound heterozygous *MUTYH* mutations, using Fisher's Exact Test.

[†], P-values for cancer patients with heterozygous *MUTYH* mutations versus controls with heterozygous *MUTYH* mutations, using Fisher's Exact Test.

^a, both families were compound heterozygous *MUTYH* mutant.

AMS; revised Amsterdam criteria [21]; BRC; breast cancer; CRC, colorectal cancer; DGR, degree relative; FCRC, familial colorectal cancer; HBCC, hereditary breast and colorectal cancer; *MUTYH*+, number of *MUTYH* p.Tyr179Cys, p.Gly396Asp and p.Pro405Leu mutations; n.s., not significant; OR, odds ratio from two-by-two tables with 95% confidence intervals; y, years of age at diagnosis.

Table 2. Cancer histories of *MUTYH* mutant families.

The number of relatives with a particular tumor type is indicated before the tumor type, with

Family Id (Category)	<i>MUTYH</i> mutation(s)		Index	Family history of cancer		
				First DGRs	Second DGRs	Third DGRs
20090 (Polyposis)	p.Tyr179Cys	p. Pro405Leu	CRC:40	Mother CRC:81 (+/-) 1 sib CRC+pol:50+55 (+/+) 2 sibs pol	4 CRC:62/77/79/83 1 other:<55	1 BRC 1 BRC+pol 1 BRC+ 2 c 3 other:<50
53119 (Polyposis)	p.Tyr179Cys	p.Gly396Asp	CRC+pol:46	Mother other 3 sibs BRC:40/<50/63 (1 +/-) 1 sib BRC+3x CRC+pol:57		
50483 (FCRC-AMS-)	p.Gly396Asp	-	CRC+pol:62	Father other:<50 1 sib CRC:40 1 sib other+BRC:63+76 3 sib other:<50/<50/79	2 BRC:49/<60 (1 -/-) 2 other:35/46 (1 -/-)	
10075 (FCRC-AMS-)	p.Pro405Leu	-	BRC:42	Father CRC:<80 1 sib CRC:69	2 BRC:33/56 1 BRC+other:56+58 (-/-) 2 CRC:<77/86 1 other:60	3 BRC:43/4
54058 (FCRC-AMS-)	p.Gly396Asp	-	CRC:54	Mother CRC:90 1 sib BRC:<57	1 CRC:<71 1 other	
15138 (HBCC)	p.Pro405Leu	-	BRC:41	Father CRC:52	1 BRC:70 1 CRC:<72 1 other:85	3 BRC:45/5
57466 (HBCC)	p.Tyr179Cys	-	BRC:43	2 sibs BRC:44/46 (1 -/-) 1 sib CRC:26	1 other:<70	1 other:66
59168 (HBCC)	p.Gly396Asp	-	BRC:35	Mother BRC:48	1 CRC:49 1 other:69	1 BRC

the age at diagnosis after the semicolon, when known. Genetic screening results for *MUTYH*

p.Tyr179Cys, p.Gly396Asp and p.Pro405Leu in non-index cases are indicated between

brackets. Genotypes are indicated as +/+, +/- and -/- for compound heterozygous,

heterozygous and non-mutation carriers, respectively.

BRC, breast cancer; CRC, colorectal cancer; Pol, adenomatous polyp(s) in the colorectum;

other, cancer other than CRC or BRC; AMS-, negative for the revised Amsterdam criteria [21];

FCRC, familial colorectal cancer; HBCC, hereditary breast and colorectal cancer; DGR,

degree relative; y, years of age at diagnosis.

Table 3. Prevalence of *MUTYH* p.Tyr179Cys, p.Gly396Asp and p.Pro405Leu mutations among 138 Dutch non-polyposis families with breast cancer and colorectal cancer.

Cancer families were classified into three categories, including families with at least two more colorectal cancer patients than breast cancer patients (CRC patients > BRC patients), families with equal numbers (plus or minus one) of colorectal cancer patients and breast cancer

patients (CRC patients = BRC patients), and families with at least two more breast cancer patients than colorectal cancer patients (CRC patients < BRC patients). *MUTYH*⁺, number of *MUTYH* p.Tyr179Cys, p.Gly396Asp and p.Pro405Leu mutations.

Classification of families	<i>MUTYH</i> +/Total tested (%)
Families with CRC patients > BRC patients	1/14 (7.1)
Families with CRC patients = BRC patients	1/55 (1.8)
Families with CRC patients < BRC patients	4/69 (5.8)