ONCOLOGY LETTERS 15: 6715-6726, 2018

Mismatch repair single nucleotide polymorphisms and thyroid cancer susceptibility

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Received September 13, 2017; Accepted December 14, 2017

DOI: 10.3892/ol.2018.8103

Abstract. Thyroid cancer (TC) is the most common endocrine malignancy and its incidence continues to rise worldwide. Ionizing radiation exposure is the best established etiological factor. Heritability is high; however, despite valuable contribution from recent genome-wide association studies, the current understanding of genetic susceptibility to TC remains limited. Several studies suggest that altered function or expression of the DNA mismatch repair (MMR) system may contribute to TC pathogenesis. Therefore, the present study aimed to evaluate the potential role of a panel of MMR single nucleotide polymorphisms (SNPs) on the individual susceptibility to well-differentiated TC (DTC). A case-control study was performed involving 106 DTC patients and 212 age- and gender-matched controls, who were all Caucasian Portuguese. Six SNPs present in distinct MMR genes (*MLH1* rs1799977, *MSH3* rs26279, *MSH4* rs5745325, *PMS1* rs5742933, *MLH3* rs175080 and *MSH6* rs1042821) were genotyped through TaqMan® assays and genotype-associated risk estimates were calculated. An increased risk was observed in *MSH6* rs1042821 variant homozygotes [adjusted odds ratio (OR)=3.42, 95% CI: 1.04-11.24, P=0.04, under the co-dominant model; adjusted OR=3.84, 95% CI: 1.18-12.44, P=0.03, under the recessive model]. The association was especially evident for the follicular histotype and female sex. The association was also apparent when *MSH6* was analysed in combination with other MMR SNPs such as *MSH3* rs26279. Interestingly, two other SNP combinations, both containing the *MSH6* heterozygous genotype, were associated with a risk reduction, suggesting a protective effect for these genotype combinations. These data support the idea that MMR SNPs such as *MSH6* rs1042821, alone or in combination, may contribute to DTC susceptibility. This is coherent with the limited evidence available. Nevertheless, further studies are needed to validate these findings and to establish the usefulness of these SNPs as genetic susceptibility biomarkers for DTC so that, in the near future, cancer prevention policies may be optimized under a personalized medicine perspective.

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Key words: *MSH6*, DNA repair, mismatch repair, single nucleotide polymorphism, genetic susceptibility, thyroid cancer

Introduction

Despite accounting for only ~2% of all human cancers, thyroid cancer (TC) is the most common endocrine malignancy. Its incidence continues to rise worldwide, being one of the cancers with the highest incidence among adolescent and young adults (ages 15-39 years) and three times more frequent in women than in men (1,2). TC is usually classified with respect to histological and clinical criteria: Papillary and follicular TC, representing 70-80% and 10-20% of cases, respectively, are the two most common varieties. Both tend to grow slowly and are often considered together as well-differentiated TC (DTC) (1,3).

DTC is generally accepted as a multifactorial disease (3). Among the several risk factors suggested to contribute to DTC, exposure to ionizing radiation (IR) remains the best-established one (1,4). Heritability is high (familial risk is one of the highest among cancers not showing typical Mendelian inheritance), suggesting that genetic factors (most likely, multiple common low-penetrance or rare moderate-penetrance alleles) strongly contribute to DTC predisposition (5). Much effort has been made to identify such susceptibility variants. The most robust evidence is for markers at 9q22.33 (*FOXE1*), 14q13.3 (*NKX2-1*), 2q35 (*DIRC3*) and 8p12 (*NRG1*), as variants in

these regions have been repeatedly associated with DTC through several genome-wide association studies (GWASs), confirmed in follow-up studies and independently replicated across different populations (6-12). Additional markers have recently been suggested (8,10-13) but still require confirmation and replication. Overall, the number of confirmed GWAS-proposed DTC risk alleles is still very limited (14) and, more importantly, explains only a relatively small proportion of the estimated heritability of DTC (11,15,16).

Multiple germ-line single nucleotide polymorphisms (SNPs) within genes involved in critical cellular processes-e.g., DNA repair, cell-cycle control and apoptosis, intracellular signalling, endobiotic or xenobiotic metabolism, thyroid physiology-have also been associated with DTC susceptibility through candidate-gene association studies (CGASs) [reviewed in (5,17)]. While most of these findings have not been properly replicated, some could, as recently demonstrated (14), represent true associations with DTC. The identification of additional variants potentially involved in DTC susceptibility may explain part of the missing heritability of the disease and is therefore highly desirable. Considering the important role that DNA-damaging agents such as IR play in DTC aetiology, DNA repair SNPs would be particularly interesting candidates. Many, across the main DNA repair pathways-BER (18,19), NER (20,21), NHEJ (22,23) and HR (24-26)-have already been associated with DTC. To our knowledge, DNA mismatch repair (MMR) SNPs have not yet been investigated.

The MMR pathway plays a crucial role in post-replication repair: It recognizes base-base mispairs and insertion/deletion loops that, in spite of the proofreading function of DNA polymerases, inescapably arise during replication. MMR thus prevents base substitutions or repeat sequence instability, greatly increasing DNA replication fidelity and safeguarding genomic integrity (27). MMR also participates, among other cellular processes (e.g., mitotic and meiotic recombination, immunoglobulin class switching), in the recognition of DNA damage induced by genotoxic chemicals, UV light, IR or oxidative stress (e.g., oxidative lesions, double strand breaks, pyrimidine dimers and inter-strand crosslinks) and subsequent repair (in cooperation with other repair pathways) or damage-induced cytotoxicity (downstream signalling for cell cycle arrest and apoptosis) (28-30). MMR's role is therefore critical to carcinogenesis: loss of MMR (e.g., inactivating mutation) greatly increases the rate of spontaneous mutation, leading to a mutator phenotype, and results in microsatellite instability (MSI), a hallmark of MMR defects (27,29,31). Not surprisingly, heterozygous germline MMR mutations (e.g., *MLH1*, *MSH2*, *MSH6* or *PMS2*) give rise to Lynch syndrome (LS), an autosomal dominant condition (hereditary nonpolyposis colorectal cancer, HNPCC) which strongly predisposes to early-onset colorectal cancer (CRC) and several extracolonic tumours, all typically presenting MSI. MMR mutations and epigenetic silencing (e.g., *MLH1* promoter methylation) are also being increasingly implicated in a growing range of tumours (27,31,32).

Interestingly, MMR mutations are increasingly being detected in TC cases (33,34) [mutation frequency correlating with progression from papillary to more aggressive TC phenotypes (35)] and TC, despite not being part of the usual LS tumour spectrum, has been incidentally observed among LS patients (36-40). The notion that MMR deficiency may contribute to TC pathogenesis and/or progression is biologically plausible since the MMR pathway is involved in the repair and damage response to IR-induced lesions such as 8-oxoGuanine (29). Supplementary evidence (reviewed in (41) further supports this hypothesis: 1) *MLH1* promoter methylation occurs in TC and is associated with lymph node metastasis and *BRAF* mutation; 2) High levels of MSI have been reported in DTC; and 3) altered MLH1, PMS1 and MSH2 expression has been reported in TC.

As such, it is possible that MMR pathway SNPs, through interference with DNA damage response and/or repair capacity in thyroid cells, could contribute to DTC susceptibility. Since this hypothesis has not yet been explored, we undertook a hospital-based case-control study in a Caucasian Portuguese population, to evaluate the potential modifying role of a panel of SNPs in MMR genes on the individual susceptibility to non-familial DTC. Identifying SNPs which may serve as DTC susceptibility biomarkers may contribute to the identification of individuals who are at increased risk for DTC and, eventually, the optimization of cancer prevention procedures.

Materials and methods

Ethical statement. This study was approved by the local ethics committees of the involved institutions and carried out in compliance with the Helsinki Declaration. At recruitment, written informed consent was obtained from each study subject and anonymity was guaranteed.

Study subjects. A total of 318 participants, all of which Caucasian Portuguese, were enrolled in this study: 106 histologically confirmed DTC patients subject to Iodine-131 treatment in the Department of Nuclear Medicine of the Portuguese Oncology Institute, Lisbon, Portugal and 212 age (±2 years) and gender-matched controls (two for each case), selected from unrelated subjects who were seeking healthcare for non-neoplasic pathology at São Francisco Xavier Hospital, Lisbon, Portugal. For controls, age at diagnosis was defined as the matched case age of diagnosis. The recruitment of both patients and controls was based on previously described (21) inclusion and exclusion criteria. At recruitment, a standard questionnaire was administered through face-to-face interviews by trained interviewers to obtain information on demographic characteristics (e.g., gender, age, occupation), family history of cancer, lifestyle habits (e.g., smoking, alcohol drinking) and IR exposure. According to the information collected, none of the study participants had been previously exposed to relevant (i.e. other than that from natural and standard diagnostic sources) levels of ionizing radiation (from therapeutic or occupational sources, e.g. none of the study participants worked or lived nearby a nuclear power plant). Detailed clinical and pathological investigation was also performed. For the purpose of smoking status, former smokers who gave up smoking either 2 years before DTC diagnosis or 2 years before their inclusion as controls were considered as non-smokers. The participation rate was 95% and blood samples were available for all subjects.

Table I. Selected SNPs and detailed information on the corresponding base and amino acid exchanges, MAF and TaqMan® assay used for genotyping.

Gene	Location	db SNP ID (rs no.)[a]	Base change	Aminoacid change	MAF (%)[a]	TaqMan® assay
MLH1	3p22.2	rs1799977	A→G	Ile219Val	13.0	C__1219076_20
MSH3	5q14.1	rs26279	A→G	Thr1045Ala	28.0	C__800002_1_
MSH4	1p31.1	rs5745325	G→A	Ala97Thr	21.3	C__3286081_10
PMS1	2q32.2	rs5742933	G→C	-[b]	21.9	C__29329633_10
MLH3	14q24.3	rs175080	G→A	Pro844Leu	36.4	C__1082805_10
MSH6	2p16.3	rs1042821	C→T	Gly39Glu	20.1	C__8760558_10

[a]According to http://www.ncbi.nlm.nih.gov/projects/SNP/ (Accessed February 15, 2017). [b]SNP located on 5'UTR. MAF, minor allele frequency; SNP, single nucleotide polymorphism.

SNP selection. Using the publicly available NCBI SNP database (http://www.ncbi.nlm.nih.gov/snp/, accessed February 15, 2017), a comprehensive set of potentially functional SNPs covering the MMR pathway were selected for genotyping. In order to be eligible, SNPs had to i) alter the amino acid sequence (missense SNPs); ii) exhibit minor allele frequency (MAF) greater than 0.10; and iii) have been previously referred to in MEDLINE (https://www.ncbi.nlm.nih.gov/pubmed/, accessed February 15, 2017). A total of five SNPs, specifically, rs1799977 (*MLH1*), rs26279 (*MSH3*), rs5745325 (*MSH4*), rs175080 (*MLH3*) and rs1042821 (*MSH6*), fulfilled these criteria and were thus analysed. In addition, since no *PMS1* SNP fulfilled all of these criteria, rs5742933- a common (MAF >0.10) 5'UTR SNP which is located within the *PMS1* promoter region (potentially regulatory role on transcription) and is the most frequently quoted *PMS1* SNP- was also included in the study. Table I summarizes the genomic location, base and amino acid exchange and MAF of the selected SNPs.

DNA extraction and genotyping. After informed consent, peripheral venous blood samples from each study subject were collected into 10 ml heparinised tubes and stored at -80˚C. Genomic DNA was extracted from these samples by using the commercially available QIAamp® DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocols. DNA extracts were kept at -20˚C until analysis.

In order to assure uniformity in DNA content (2.5 ng/μl) prior to genotyping, DNA quantity was assessed fluorimetrically in all samples using the Quant-iT™ Picogreen® dsDNA Assay kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and a Zenyth 3100 plate reader (Anthos Labtec Instruments, Salzburg, Austria).

SNP genotyping was carried out using the Taqman® allelic discrimination assay on a 96-well ABI 7300 Real Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.), following the manufacturer's instructions. Commercial pre-designed assay primers and probes, purchased from Applied Biosystems; Thermo Fisher Scientific, Inc., were used for every SNP and are listed in Table I. The amplification conditions consisted of an initial activation step (10 min, 95˚C), followed by ≥40 amplification cycles of denaturation (15 sec, 92˚C) and annealing/extension (60 sec, 60˚C). The fluorescence intensity emitted by VIC and FAM dyes in each well was detected (60 sec) and analysed with Applied Biosystems sequence detection software (System SDS version 1.3.1).

To assure accuracy of the genotyping and avoid variant misclassification, four negative controls (wells containing no DNA) were included in each plate. Genotyping of inconclusive samples was repeated. Also, for quality control, 10-15% of the samples were randomly selected and run in duplicates. 100% concordance between experiments was observed.

Statistical analysis. Prior to analysis, an exact probability test available in SNPStats software (42) was used to check whether genotype distributions for each studied SNP deviated significantly from Hardy-Weinberg equilibrium (HWE).

Since all variables considered were categorical or categorized (e.g., age), descriptive statistics were presented as frequencies and percentages.

The distribution of demographic variables such as gender, age group and smoking status and of genotype frequencies was compared between groups through Chi-square or two-sided Fisher's exact test for 2x2 or 2x3 contingency tables, respectively.

For all elected SNPs, genotype-associated risk of DTC was estimated by binary logistic regression analysis and expressed as both crude and adjusted odds ratios (OR) and 95% confidence intervals (CI). Risk estimates were calculated under codominant, dominant, recessive and log-additive genetic models. Adjustment, when performed, included terms for gender (male/female), age group (<30, 30-49, 50-69 and ≥70 years) and smoking habits (smokers/non-smokers). The most common homozygous genotype, female gender, lower age group and non-smoking status were taken as reference for the purpose of such calculations. The remaining information that was collected on demographic characteristics (e.g. occupation), family history of cancer, lifestyle habits (e.g. alcohol drinking) and prior IR exposure was not suitable for rigorous quantitative transformation and, therefore, not included in the adjustment.

Stratified analysis according to histological type of tumour (papillary or follicular TC), gender and age was also performed. Additionally, we conducted a genotype interaction analysis (combination of alleles) in order to investigate the

combined effect of different pairs of SNPs on DTC risk. All possible combinations were analysed. For each pair of SNPs, the combination of the most common homozygous genotypes of each individual SNP was taken as the reference category. Paired genotypes with frequency <5% in the control group were pooled together.

Finally, the chromosomic location of the variants included in this study was compared to that of DTC markers previously reported in GWAS. Linkage disequilibrium between co-localized variants in European populations was verified *in silico* through the use of LDLink (43), a publicly available web-based application that uses Phase 3 haplotype data from the 1,000 Genomes Project to calculate pairwise LD between user-input variants in different population groups.

This was a 'proof of concept' study to ascertain whether MMR variants might be linked to DTC. Bonferroni adjustment was not used because it is too conservative. Also, the complement of the false negative rate β to compute the power of a test (1-β) was not taken into account at this stage since further studies with more patients and controls should be undertaken to change over this preliminary study into a confirmatory positive one.

The statistical analysis was done with SPSS 22.0 (IBM SPSS Statistics for Windows, version 22.0; IBM Corp, Armonk, NY, USA) except for HWE deviation assessment, MAF calculations, haplotype estimation and linkage disequilibrium (LD) analysis which were performed using the SNPstats Software (42). Two-tailed P<0.05 was considered to indicate a statistically significant difference.

Results

The demographic characteristics of the 106 DTC cases and their 212 age and gender-matched controls are depicted in Table II. The mean age for each group was 52 years (range 19-77 in the patient group and 18-77 in the control group). Female patients significantly outnumbered male patients, in accordance with the worldwide estimation for gender distribution in DTC (1,2). A total of 11.3% of patients were categorized as smokers. No statistically significant difference between groups was observed concerning age distribution, gender and smoking habits. Regarding DTC histological classification, 78 (73.6%) patients were diagnosed as of the papillary type, while 28 (26.4%) were diagnosed as of the follicular type. All cancer patients were incident cases and none of the controls had a family history of cancer.

Table III summarizes the results for MAF, genotypic frequencies and crude/adjusted ORs of the six MMR pathway SNPs selected in our study. The genotype distributions of the studied SNPs were in HWE (P≥0.05), in both case and control groups. No relevant LD was observed between the studied SNPs (data not shown). When comparing, for each of the studied SNPs, the genotype frequency distribution between cases and controls, a significant difference was observed only for *MSH6* rs1042821 (P=0.04, on the codominant and recessive models). Statistical significance was not attained when assuming a dominant model of inheritance (P=0.54). No additional significant differences were found, irrespective of the model of inheritance assumed. When performing logistic regression analysis, a significant DTC risk increase was observed in *MSH6* rs1042821 variant allele homozygotes, after adjustment for age, gender and smoking status (Glu/Glu vs. Gly/Gly: adjusted OR=3.42, 95% CI: 1.04-11.24, P=0.04; Glu/Glu vs. Gly/Gly+Gly/Glu: adjusted OR=3.84, 95% CI: 1.18-12.44, P=0.03). This association was also apparent without covariate adjustment when assuming a recessive model of inheritance (Glu/Glu vs. Gly/Gly+Gly/Glu: OR=3.35, 95% CI: 1.07-10.50, P=0.04). No significant associations with DTC risk were observed for the remaining SNPs analysed in this study, irrespective of the model assumed.

Table II. General characteristics for the DTC case (n=106) and control (n=212) populations.

Characteristics	Controls n (%)	Cases n (%)	P-value[c]
Gender			
Male	31 (14.6)	16 (15.1)	1.00
Female	181 (85.4)	90 (84.9)	
Age[a,b]			
<30	10 (4.7)	4 (3.8)	0.98
30-49	75 (35.4)	38 (35.8)	
50-69	98 (46.2)	49 (46.2)	
≥70	29 (13.7)	15 (14.2)	
Smoking habits			
Non-smokers	174 (82.1)	94 (88.7)	0.19
Smokers	36 (17.0)	12 (11.3)	
Missing	2 (0.9)	0 (0.0)	

[a]Age of diagnosis, for cases. [b]Age at the time of diagnosis of the matched case, for controls. [c]P-value for cases vs. control group determined by two-sided Fisher's exact test (gender, smoking habits) or χ^2 test (age). DTC, well-differentiated thyroid cancer.

Since DTC comprises two distinct histological types (papillary and follicular), affects women more than men and is the most incident malignancy in the 15-39 years age group (1,2), patients and controls were stratified on the basis of these criteria, i.e., histological tumour type, gender and age, in order to identify any subgroup-specific risk association. As shown in Table IV, stratification of subjects according to histological criteria showed that the association between the homozygous variant genotype of *MSH6* rs1042821 and DTC risk, observed in the complete set of patients, was also present in the follicular subset (adjusted OR=20.98, 95% CI: 1.08-406.53, P=0.04, under the co-dominant model; adjusted OR=23.70, 95% CI: 1.25-449.32, P=0.04, under the recessive model) but absent from the papillary subset, suggesting a histological type-specific SNP effect. Also in the follicular subset, a significant difference in the genotype frequency distribution of *MLH3* rs175080 was observed (P=0.04, in the dominant model, data not shown). On binary logistic regression analysis, significantly increased follicular TC risk was observed in *MLH3* rs175080 variant allele carriers (OR=3.95, 95% CI: 1.05-14.81, P=0.04). After gender stratification (Table IV), the frequency distribution of *MSH6* rs1042821 genotypes differed significantly between female DTC patients and their age and gender-matched controls (P=0.02, in the codominant model,

Table III. Genotype distribution in case (n=106) and control (n=212) populations and associated DTC risk (crude and adjusted ORs).

	MAF		Genotype frequency				
Genotype	Controls	Cases	Controls n (%)	Cases n (%)	P-value[a]	OR (95% CI)	Adjusted OR (95% CI)[b]
MLH1 rs1799977			212 (100)	105 (100)			
Ile/Ile	G: 0.34	G: 0.30	93 (43.9)	48 (45.7)	0.42	1 (Reference)[d]	1 (Reference)[d]
Ile/Val			95 (44.8)	50 (47.6)		1.02 (0.63-1.66)	1.02 (0.62-1.68)
Val/Val			24 (11.3)	7 (6.7)		0.57 (0.23-1.41)	0.56 (0.22-1.40)
Dominant model			119 (56.1)	57 (54.3)	0.81	0.93 (0.58-1.49)	0.93 (0.58-1.50)
Recessive model			24 (11.3)	7 (6.7)	0.23	0.56 (0.23-1.34)	0.55 (0.23-1.34)
Log-additive model			-	-	-	0.86 (0.59-1.23)	0.85 (0.59-1.24)
MSH3 rs26279			211 (100)	105 (100)			
Thr/Thr	G: 0.35	G: 0.33	93 (44.1)	48 (45.7)	0.89	1 (Reference)[d]	1 (Reference)[d]
Thr/Ala			90 (42.7)	45 (42.9)		0.97 (0.59-1.60)	0.94 (0.57-1.56)
Ala/Ala			28 (13.3)	12 (11.4)		0.83 (0.39-1.78)	0.80 (0.37-1.72)
Dominant model			118 (55.9)	57 (54.3)	0.81	0.94 (0.59-1.50)	0.91 (0.56-1.46)
Recessive model			28 (13.3)	12 (11.4)	0.72	0.84 (0.41-1.73)	0.82 (0.40-1.70)
Log-additive model			-	-	-	0.93 (0.66-1.31)	0.91 (0.64-1.28)
MSH4 rs5745325			212 (100)	106 (100)			
Ala/Ala	A: 0.33	A: 0.27	97 (45.8)	57 (53.8)	0.38	1 (Reference)[d]	1 (Reference)[d]
Ala/Thr			91 (42.9)	40 (37.7)		0.75 (0.46-1.23)	0.75 (0.45-1.23)
Thr/Thr			24 (11.3)	9 (8.5)		0.64 (0.28-1.47)	0.64 (0.28-1.48)
Dominant model			115 (54.2)	49 (46.2)	0.19	0.73 (0.45-1.16)	0.72 (0.45-1.16)
Recessive model			24 (11.3)	9 (8.5)	0.56	0.73 (0.33-1.63)	0.72 (0.32-1.63)
Log-additive model			-	-	-	0.78 (0.54-1.11)	0.78 (0.54-1.11)
PMS1 rs5742933			212 (100)	104 (100)			
G/G	C: 0.18	C: 0.17	144 (67.9)	73 (70.2)	0.90	1 (Reference)[d]	1 (Reference)[d]
G/C			58 (27.4)	27 (26.0)		0.92 (0.54-1.57)	0.88 (0.51-1.51)
C/C			10 (4.7)	4 (3.8)		0.79 (0.24-2.60)	0.76 (0.23-2.60)
Dominant model			68 (32.1)	31 (29.8)	0.70	0.90 (0.54-1.50)	0.86 (0.51-1.45)
Recessive model			10 (4.7)	4 (3.8)	1.00	0.81 (0.25-2.64)	0.80 (0.24-2.67)
Log-additive model			-	-	-	0.90 (0.59-1.38)	0.88 (0.57-1.35)
MLH3 rs175080			212 (100)	106 (100)			
Pro/Pro	A: 0.46	A: 0.51	60 (28.3)	22 (20.8)	0.34	1 (Reference)[d]	1 (Reference)[d]
Pro/Leu			109 (51.4)	59 (55.7)		1.48 (0.83-2.64)	1.50 (0.83-2.71)
Leu/Leu			43 (20.3)	25 (23.6)		1.59 (0.79-3.17)	1.60 (0.79-3.22)
Dominant model			152 (71.7)	84 (79.2)	0.17	1.51 (0.86-2.63)	1.53 (0.87-2.69)
Recessive model			43 (20.3)	25 (23.6)	0.56	1.21 (0.69-2.12)	1.21 (0.69-2.12)
Log-additive model			-	-	-	1.26 (0.90-1.77)	1.26 (0.89-1.78)
MSH6 rs1042821			210 (100)	106 (100)			
Gly/Gly	T: 0.21	T: 0.22	127 (60.5)	68 (64.2)	**0.04**[c]	1 (Reference)[d]	1 (Reference)[d]
Gly/Glu			78 (37.1)	30 (28.3)		0.72 (0.43-1.20)	0.73 (0.43-1.23)
Glu/Glu			5 (2.4)	8 (7.5)		2.99 (0.94-9.49)	**3.42 (1.04-11.24)**[c]
Dominant model			83 (39.5)	38 (35.8)	0.54	0.86 (0.53-1.39)	0.87 (0.54-1.43)
Recessive model			5 (2.4)	8 (7.5)	**0.04**[c]	**3.35 (1.07-10.50)**[c]	**3.84 (1.18-12.44)**[c]
Log-additive model			-	-	-	1.05 (0.70-1.57)	1.08 (0.71-1.63)

[a]P-value for cases vs. control group determined by two-sided Fisher's exact test (whenever 2x2 contingency tables are possible) or χ^2 test (remaining cases). [b]ORs were adjusted for gender (male and female), age (<30, 30-49, 50-69, ≥70 years) and smoking status (non-smoker and smoker). [c]Significant results (P<0.05) highlighted in bold. [d]The reference comparator for OR calculations. DTC, well-differentiated thyroid cancer; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval.

Table IV. Genotype distribution in the case population (n=106) and associated DTC risk (crude and adjusted ORs), after stratification according to histological type, gender and age.[a]

A, Histological type

	Papillary carcinoma			Follicular carcinoma		
Genotype	n (%)	OR (95% CI)	Adjusted OR (95% CI)[b]	n (%)	OR (95% CI)	Adjusted OR (95% CI)[b]
MSH6 rs1042821	78 (100)			28 (100)		
Gly/Gly	49 (62.8)	1 (reference)[e]	1 (reference)[e]	19 (67.9)	1 (reference)[e]	1 (reference)[e]
Gly/Glu	24 (30.8)	0.74 (0.41-1.32)	0.74 (0.41-1.35)	6 (21.4)	0.65 (0.22-1.91)	0.76 (0.24-2.35)
Glu/Glu	5 (6.4)	2.30 (0.59-8.95)	2.47 (0.61-9.89)	3 (10.7)	5.84 (0.57-60.03)	**20.98 (1.08-406.53)[c]**
Dominant model	29 (37.2)	0.83 (0.48-1.46)	0.85 (0.48-1.49)	9 (32.1)	0.92 (0.35-2.43)	1.10 (0.39-3.07)
Recessive model	5 (6.4)	2.57 (0.67-9.85)	2.74 (0.69-10.84)	3 (10.7)	6.60 (0.65-66.63)	**23.70 (1.25-449.32)[c]**
Log-additive model	-	0.98 (0.61-1.59)	1.00 (0.62-1.62)	-	1.24 (0.57-2.68)	1.58 (0.66-3.75)
MLH3 rs175080	78 (100)			28 (100)		
Pro/Pro	19 (24.4)	1 (reference)[e]	1 (reference)[e]	3 (10.7)	1 (reference)[e]	1 (reference)[e]
Pro/Leu	42 (53.8)	1.13 (0.59-2.19)	1.17 (0.60-2.27)	17 (60.7)	3.78 (0.97-14.79)	3.61 (0.88-14.85)
Leu/Leu	17 (21.8)	1.17 (0.53-2.61)	1.20 (0.54-2.68)	8 (28.6)	4.36 (0.95-20.04)	4.29 (0.89-20.78)
Dominant model	59 (75.6)	1.14 (0.61-2.14)	1.18 (0.62-2.22)	25 (89.3)	**3.95 (1.05-14.81)[c]**	3.81 (0.97-14.95)
Recessive model	17 (21.8)	1.08 (0.56-2.10)	1.08 (0.56-2.10)	8 (28.6)	1.64 (0.57-4.69)	1.67 (0.55-5.02)
Log-additive model	-	1.09 (0.73-1.62)	1.10 (0.74-1.63)	-	1.93 (0.97-3.86)	1.93 (0.93-4.01)

B, Gender

	Male			Female		
Genotype	n (%)	OR (95% CI)	Adjusted OR (95% CI)[b]	n (%)	OR (95% CI)	Adjusted OR (95% CI)[b]
MSH6 rs1042821	16 (100)			90 (100)		
Gly/Gly	11 (68.8)	1 (reference)[e]	1 (reference)[e]	57 (63.3)	1 (reference)[e]	1 (reference)[e]
Gly/Glu	4 (25.0)	0.86 (0.21-3.54)	0.96 (0.20-4.52)	26 (28.9)	0.70 (0.41-1.22)	0.70 (0.40-1.22)
Glu/Glu	1 (6.3)	0.86 (0.07-10.66)	1.08 (0.07-16.53)	7 (7.8)	**4.42 (1.10-17.75)[c]**	**4.78 (1.17-19.56)[c]**
Dominant model	5 (31.3)	0.86 (0.23-3.19)	0.98 (0.23-4.24)	33 (36.7)	0.86 (0.51-1.44)	0.86 (0.51-1.45)
Recessive model	1 (6.3)	0.90 (0.08-10.77)	1.09 (0.08-15.61)	7 (7.8)	**5.00 (1.26-19.84)[c]**	**5.42 (1.34-21.92)[c]**
Log-additive model	-	0.90 (0.33-2.48)	1.00 (0.32-3.14)	-	1.08 (0.69-1.69)	1.09 (0.70-1.71)

C, Age

	<50 years			≥50 years		
Genotype	n (%)	OR (95% CI)	Adjusted OR (95% CI)[b]	n (%)	OR (95% CI)	Adjusted OR (95% CI)[b]
MSH6 rs1042821	42 (100)			64 (100)		
Gly/Gly	29 (69.0)	1 (reference)[e]	1 (reference)[e]	39 (60.9)	1 (reference)[e]	1 (reference)[e]
Gly/Glu	12 (28.6)	0.56 (0.25-1.27)	0.56 (0.25-1.26)	18 (28.1)	0.84 (0.43-1.64)	0.86 (0.44-1.70)
Glu/Glu	1 (2.4)	0.31 (0.03-2.79)	0.32 (0.04-2.93)	7 (10.9)	-[d]	-[d]
Dominant model	13 (31.0)	0.53 (0.24-1.16)	0.53 (0.24-1.17)	25 (39.1)	1.17 (0.63-2.17)	1.21 (0.64-2.27)
Recessive model	1 (2.4)	0.38 (0.04-3.37)	0.40 (0.04-3.58)	7 (10.9)	-[d]	-[d]
Log-additive model	-	0.56 (0.28-1.12)	0.56 (0.28-1.12)	-	1.57 (0.93-2.66)	1.63 (0.95-2.79)

[a]Only SNPs presenting significant findings are shown. [b]ORs were adjusted for gender (male and female), age (<30, 30-49, 50-69, and ≥70 years), and smoking status (non-smoker and smoker). [c]Significant results (P<0.05) highlighted in bold. [d]Genotype not found in the corresponding controls. [e]The reference comparator for OR calculations. DTC, well-differentiated thyroid cancer; SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

Table V. Two-way SNP interactions: distribution of combined genotypes in the case (n=106) and control (n=212) populations and associated DTC risk (crude and adjusted ORs).[a]

Genotype	Genotype frequency			DTC risk			
	Controls n (%)	Cases n (%)	P-value[b]	OR (95% CI)	P-value[b]	Adjusted OR (95% CI)[c]	P-value[b]
MSH6 rs1042821-*MSH3* rs26279	209 (100)	105 (100)	0.167				
Gly/Gly-Thr/Thr	59 (28.2)	29 (27.6)		1 (reference)[e]		1 (reference)[e]	
Gly/Gly-Thr/Ala	56 (26.8)	32 (30.5)		1.16 (0.62-2.16)	0.64	1.18 (0.63-2.20)	0.62
Gly/Glu-Thr/Thr	31 (14.8)	16 (15.2)		1.05 (0.50-2.22)	0.90	1.14 (0.53-2.43)	0.74
Gly/Glu-Thr/Ala	33 (15.8)	10 (9.5)		0.62 (0.27-1.42)	0.26	0.60 (0.26-1.39)	0.23
Gly/Gly-Ala/Ala	11 (5.3)	7 (6.7)		1.30 (0.46-3.69)	0.63	1.26 (0.44-3.62)	0.67
Glu/Glu-Thr/Thr	5 (2.4)	8 (7.6)		3.26 (0.98-10.84)	0.05	**3.81 (1.11-13.13)[d]**	**0.03[d]**
Glu/Glu-Thr/Ala							
Glu/Glu-Ala/Ala							
Gly/Glu-Ala/Ala	14 (6.7)	3 (2.9)		0.44 (0.12-1.64)	0.22	0.42 (0.11-1.59)	0.20
MLH3 rs175080-*MSH6* rs1042821	210 (100)	106 (100)	**0.032[d]**				
Pro/Pro-Gly/Gly	32 (15.2)	19 (17.9)		1 (reference)[e]		1 (reference)[e]	
Pro/Pro-Gly/Glu	26 (12.4)	2 (1.9)		**0.13 (0.03-0.61)[d]**	**0.01[d]**	**0.11 (0.02-0.53)[d]**	**0.01[d]**
Pro/Leu-Gly/Gly	71 (33.8)	36 (34.0)		0.85 (0.43-1.71)	0.66	0.81 (0.40-1.65)	0.56
Pro/Leu-Gly/Glu	35 (16.7)	19 (17.9)		0.91 (0.41-2.03)	0.83	0.94 (0.41-2.13)	0.88
Pro/Pro-Glu/Glu	5 (2.4)	8 (7.5)		2.70 (0.77-9.44)	0.12	3.09 (0.85-11.27)	0.09
Pro/Leu-Glu/Glu							
Leu/Leu-Glu/Glu							
Leu/Leu-Gly/Gly	24 (11.4)	13 (12.3)		0.91 (0.38-2.20)	0.84	0.83 (0.34-2.03)	0.68
Leu/Leu-Gly/Glu	17 (8.1)	9 (8.5)		0.89 (0.33-2.39)	0.82	0.89 (0.33-2.43)	0.82
MSH4 rs5745325-*MSH6* rs1042821	210 (100)	106 (100)	0.149				
Ala/Ala-Gly/Gly	53 (25.2)	36 (34.0)		1 (reference)[e]		1 (reference)[e]	
Ala/Ala-Gly/Glu	41 (19.5)	20 (18.9)		0.72 (0.36-1.42)	0.34	0.74 (0.37-1.47)	0.39
Ala/Thr-Gly/Gly	59 (28.1)	26 (24.5)		0.65 (0.35-1.21)	0.18	0.66 (0.35-1.23)	0.19
Ala/Thr-Gly/Glu	30 (14.3)	7 (6.6)		**0.34 (0.14-0.87)[d]**	**0.02[d]**	**0.35 (0.14-0.88)[d]**	**0.03[d]**
Ala/Ala-Glu/Glu	12 (5.7)	11 (10.4)		1.35 (0.54-3.39)	0.52	1.43 (0.56-3.66)	0.45
Ala/Thr-Glu/Glu							
Thr/Thr-Gly/Glu							
Thr/Thr-Glu/Glu							
Thr/Thr-Gly/Gly	15 (7.1)	6 (5.7)		0.59 (0.21-1.66)	0.32	0.60 (0.21-1.70)	0.33

[a]Only combined genotypes presenting significant findings are shown. [b]P-value for cases vs. control group determined by two-sided Fisher's exact test (whenever 2x2 contingency tables are possible) or χ^2 test (remaining cases). [c]ORs were adjusted for gender (male and female), age (<30, 30-49, 50-69, ≥70 years) and smoking status (non-smoker and smoker). [d]Significant results (P<0.05) highlighted in bold. [e]The reference comparator for OR calculations. DTC, well-differentiated thyroid cancer; SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

data not shown). Also, as depicted in Table IV, the homozygous variant genotype of this SNP was found to confer increased DTC risk in females only, under both the co-dominant (OR=4.42, 95% CI: 1.10-17.75, P=0.04 and adjusted OR=4.78, 95% CI: 1.17-19.56, P=0.03) and the recessive model (OR=5.00, 95% CI: 1.26-19.84, P=0.02 and adjusted OR=5.42, 95% CI: 1.34-21.92, P=0.02), supporting the idea that this polymorphism might influence DTC susceptibility, particularly in women. The study population was also stratified according to the age of diagnosis (Table IV). In order to avoid excessively low numbers in each strata, only two groups were formed: <50 and ≥50 years. In the elderly group (≥50 years), a highly significant difference in the frequency distribution of *MSH6* rs1042821 genotypes was observed between DTC patients and the corresponding controls (P=0.001 in the codominant model, data not shown). Unfortunately, no *MSH6* rs1042821

homozygous variant individuals ≥50 years were observed in the control group, limiting OR calculations and subsequent analysis for this SNP. Further analysis of our study subjects after histological type, gender and age stratification revealed no other significant correlations between the analysed SNPs and DTC risk.

Proteins that participate in a common DNA repair pathway functionally interact with each other, establishing ground for additive or even multiplicative effects of different SNPs of the same pathway on DNA repair activity and, hence, cancer risk. This has been previously demonstrated for other DNA repair pathways (16,44,45) and, most likely, also applies to MMR, justifying the usefulness of assessing the effect of combined genotypes on DTC risk. As detailed in Table V, the combined genotype distribution of the *MSH6* rs1042821-*MLH3* rs175080 SNP pair was significantly different in cases and controls (P=0.032). On logistic regression analysis, when *MSH6* rs1042821 and *MSH3* rs26279 were considered together, a significantly increased risk was observed in the pooled group of *MSH6* rs1042821 variant allele homozygotes, despite only after adjusting for gender, age and smoking status (adjusted OR=3.81, 95% CI: 1.11-13.13, P=0.03). Interestingly, two other *MSH6* rs1042821 genotype combinations, all involving the rs1042821 heterozygous genotype, yielded significant results in the opposite direction: a significantly decreased risk was detected in combined *MSH6* rs1042821-*MSH4* rs5745325 heterozygotes (OR=0.34, 95% CI: 0.14-0.87, P=0.02 and adjusted OR=0.35, 95% CI: 0.14-0.88, P=0.03), as well as in individuals simultaneously heterozygous for *MSH6* rs1042821 and homozygous for the common allele of *MLH3* rs175080 (OR=0.13, 95% CI: 0.03-0.61, P=0.01 and adjusted OR=0.11, 95% CI: 0.02-0.53, P=0.01). None of the remaining genotype combinations showed association with disease (data not shown).

Finally, since *MSH3* and *MLH3* (whose studied variants yielded significant associations on SNP pair analysis) are located in the same chromosomic region that DTC markers reported in prior GWAS (rs13184587 at 5q14.1 and rs10136427 at 14q24.3, respectively) (13), we used LDLink (43) to verify *in silico* any potential linkage disequilibrium relation between these MMR variants and the GWAS-suggested markers co-localized in the same chromosomic region. No linkage disequilibrium was observed between either *MSH3* rs26279 and rs13184587 or *MLH3* rs175080 and rs10136427 in European populations (data not shown).

Discussion

To our knowledge, this was the first study evaluating the potential role of MMR SNPs on DTC susceptibility in Caucasian populations.

We observed a significantly increased DTC risk in *MSH6* rs1042821 variant allele homozygotes (Glu/Glu). *MSH6* rs1042821 is probably one of the most studied SNPs of the MMR pathway and its potential association with cancer (other than TC) has previously been addressed, with inconsistent results: rs1042821 has been associated with increased CRC risk (46,47), as well as with triple negative breast cancer (TNBC) (48) and highly malignant bladder cancer (49). Contrasting results have been reported for hepatocellular (50), colorectal (51) and pancreatic cancer (52). Most studies, however, present inconclusive findings (53-57), including a recent meta-analysis (58) aggregating data from many of the above-quoted studies. It is possible that organ and population-specific characteristics (e.g., genetic background and environmental exposure) may have contributed to such diverse observations. More recently, rs1042821 has also been detected through sequencing techniques in several cancer cases (59-61) but, considering the high population frequency of this SNP, this could be merely coincidental.

The involvement of *MSH6* SNPs in cancer susceptibility (DTC, in particular) is expected for three fundamental reasons. Firstly, because it is biologically plausible: MSH6 integrates the MutSα complex, a sensor of genetic damage that, besides its role in the repair of replication errors, cooperates with other DNA repair and damage-response signalling pathways to allow for cell cycle arrest, DNA repair and/or apoptosis of genetically damaged cells. Of importance for DTC susceptibility, MutSα ensures accurate homologous recombination repair of double strand breaks and cooperates with MUTYH in the repair of 8-oxoGuanine [reviewed in (27-29)], lesions that commonly arise from IR exposure, the most well-known DTC risk factor. Secondly, because of the functional impact of *MSH6* mutations: Experimental studies in MSH6-deficient yeast, mice or human cells demonstrate that *MSH6* mutations results in partial MMR deficiency (mild mutator phenotype, characterized by weak microsatellite instability, MSI-L) and increased cancer susceptibility in animal models (27). And finally because, in the clinical context, *MSH6* mutations are associated with cancer syndromes (and TC, possibly): Inherited *MSH6* germline mutations are responsible for 7-10% of LS cases, patients presenting an atypical phenotype (lower CRC incidence-with later onset-high incidence of endometrial cancer and lower degree or absence of MSI), compared to the more frequent *MSH2* and *MLH1*-mutant LS cases (27,31,39,62). TC-despite not part of the usual LS spectrum- has been sporadically observed in LS patients harbouring *MSH2* and *MLH1* mutations (36,38-40) and, more recently, also in a *MSH6*-mutant LS case (37). *MSH6* mutations were also recently observed in both anaplastic (33) and papillary TC (34). In the latter study, *MSH6* was even the most frequently mutated gene and two of these mutations (Gly355Ser and Ala36Val) were coincidental in family-related patients, suggesting a causative association. For all of the above, it is likely that *MSH6* genetic variation contributes to TC development.

The rs1042821 SNP is a common missense variant that involves the substitution 116G>A in exon 1 of the *MSH6* gene. It results in the substitution of glutamic acid for glycine at position 39 (Gly39Glu) of the MSH6 N-terminal region (NTR), a highly disordered domain upstream of the mismatch binding domain. The importance of the MSH6 NTR is being increasingly recognized as missense mutations in this region have been associated with cancer [an exhaustive list of LS-associated mutations is available in the InSiGHT database (32)]. Interestingly, the MSH6 NTR is absent from prokaryotic MutS which, coincidentally, does not share some of the functions of eukaryotic MutSα (e.g., activation of apoptosis) (63), suggesting a critical role for this region in such processes. As extensively reviewed

in Edelbrock *et al* (28), several sequence motifs in the NTR may be of relevance to the multitude of actions performed by MSH6, including: 1) a short, conserved PCNA interacting protein (PIP) motif, located near the N-terminal extreme, that allows PCNA binding; 2) a PWWP sequence motif, distal to the PIP box, that mediates interactions with chromatin and chromatin-associating proteins; 3) a conserved motif near the NTR C-terminus, rich in positively charged amino acids that (through electrostatic attraction) contributes to nonspecific DNA binding and stabilizes the MutSα-DNA interaction (possibly modulating the residence time of MutSα at the lesion site); 4) nuclear localization sequences (NLSs, e.g., a conserved Ser-Pro-Ser sequence-amino acids 41-43-containing phosphorylated serines), that may contribute to the nuclear import of MutSα; and 5) multiple phosphorylation sites (19 out of the 23 identified in MSH6, according to the updated list at http://www.uniprot.org/uniprot/P52701), that may be involved in the post-translational regulation of MutSα stability, nuclear import and differential downstream signalling for MMR and DNA damage response. The NTR may also be responsible for other protein interactions.

Functional assays are needed to confirm if the *MSH6* rs1042821 variant affects the function of MutSα. However, given its location-in the MSH6 NTR, near a NLS containing two phosphorylation sites (Ser41 and Ser43)- it is possible that rs1042821 interferes with phosphorylation of these residues (both MAPK recognition motifs) and hence with the post-translational regulation of MutSα stability, nuclear import or activity (28). The rs1042821 SNP may also interfere with non-specific DNA binding since glutamate, contrasting with glycine, is negatively charged at physiological pH. This may hamper electrostatic attraction to the phosphate backbone of DNA, interfere with the stability of the MutSα-DNA interaction and hence decrease the residence time of MutSα at the lesion site [previously suggested to play a role in the differential regulation of the DNA repair and apoptosis signalling roles of MSH6 (63)]. In fact, increasing number of negatively charged glutamate residues within the amino acid 231-289 NTR segment of the yeast Msh6 increases mutation rates in these cells (64) and substitutions of glutamic acid for glycine, in general, can determine the formation of sterically different helical structures, polypeptide folding, and intrinsic aggregation (51). Whether this applies to *MSH6* rs1042821 remains to be established.

In our study, upon stratification, the association between the *MSH6* rs1042821 homozygous variant genotype and increased DTC risk was especially evident for the follicular histotype, female sex and, possibly, older age (≥50 years). Concerning the histological type of tumour, this contrasts with prior evidence: rs1042821 has been associated with the development of *BRAF* mutated (Val600Glu) colon tumours (54) -only in microsatellite stable (MSS), not MSI tumours- and the Val600Glu *BRAF* mutation is a hallmark of papillary, not follicular TC (3). However, as in our study, this observation resulted from stratification analysis with only a limited number of subjects in each strata [n=3 for follicular TC cases with Glu/Glu genotype in our study; n=4 for *BRAF* mutated, MSS tumours with Glu/Glu genotype in (54)]. Either observation could therefore be due to chance (type I statistical error), hampering solid conclusions. Further studies with a larger sample size are needed to clarify the relationship, if any, between rs1042821 and DTC histological type. The genotype-disease association was stronger among women, an expected finding since DTC affects women more than men (1,2), differential incidence starting with the onset of puberty and declining after menopause (65). Also, TC rates in women with breast cancer history (and vice-versa) are higher than expected (66), suggesting some common ground between these conditions. Oestrogen could be the 'missing link': Besides its well-established role in the pathogenesis of several endocrine-related cancers (e.g., breast, endometrial, ovarian) (67), oestrogen may promote the growth of TC cells and thus contribute to development and progression of DTC, through increased transcription of ERE-containing genes, activation of the MAPK and PI3K signalling pathways, modulation of the TC microenvironment or specific effects on thyroid stem and progenitor cells (65). Oestrogen has also been suggested to give rise to cancer-initiating mutations through the formation of DNA adducts and other oxidative lesions, high levels of which have been observed in women with breast, thyroid or ovarian cancer (68). On the other hand, MSH6 is increasingly being implicated in such oestrogen-associated cancers as 1) *in vitro* oestrogen exposure after catechol-O-methyltransferase inhibition increases the levels of 8-oxo-dG (69), an oxidative DNA lesion whose repair involves the MutSα complex; 2) *MSH6* mutations and reduced *MSH6* mRNA expression have been reported in breast cancer patients and breast tumour derived cell lines, respectively (70); 3) in LS patients, endometrial cancer is commonly associated with *MSH6* mutations (27,31,39,62); 4) MSH2- the binding partner of MSH6 in MutSα- is able to transactivate the oestrogen receptor α, through its MSH6 interaction domain (71); 5) several DNA repair SNPs have been associated with increased oestrogen sensitivity in the development of breast cancer (72-74). Also, we previously reported a non-significant breast cancer risk increase in rs1042821 variant allele homozygotes (53), in line with the results reported here. Overall, if oestrogen indeed contributes to DTC and MSH6 is indeed involved in oestrogen-associated cancers, it is only logical that a putative association between rs1042821 and DTC susceptibility is particularly visible in women. Finally, in the current study, considering only individuals of age ≥50 years, the rs1042821 homozygous variant genotype was detected only in DTC patients, not in controls. This may suggest that rs1042821 is associated with DTC susceptibility particularly among older individuals. This is compatible with the observation of later onset cancer in LS patients harbouring *MSH6* mutations (27,31,39). Also, in line with our results, rs1042821 has been associated with increased breast cancer risk in women of age >60 years and decreased risk in women of age ≤60 years (57). Finally, MSH6 has been demonstrated to be markedly downregulated in senescent cells (75), suggesting that MutSα activity decreases during the aging process. Since our results were based on stratified analysis, further studies are required to confirm this finding.

On paired SNP analysis, several associations were observed in our study. Most of these involve *MSH6* rs1042821, possibly reflecting an individual SNP effect. However- since MMR proteins functionally interact within the same pathway- an additive (or even multiplicative) effect with other MMR SNPs is possible. Supporting this hypothesis, several studies

have shown that, although individual susceptibility alleles may have only a modest effect, DTC risk may be substantially increased when multiple risk variants are considered together (15,16). Considering the strong genetic component of DTC susceptibility, such a role for gene-gene interactions is likely (16). One SNP combination comprising the *MSH6* rs1042821 homozygous variant genotype was associated with increased DTC risk, as expected from single SNP analysis. Interestingly, two other SNP pairs containing the *MSH6* heterozygous genotype were associated with a risk reduction. A similar non-significant trend was already evident on single SNP analysis, suggesting a protective effect for the *MSH6* rs1042821 heterozygous genotype. Prior evidence supports this hypothesis: We previously reported a breast cancer risk reduction in combined *MSH6* rs1042821 heterozygotes/*MSH3* rs26279 common allele homozygotes (53).

Other studies (50-53,57), including a recent meta-analysis by Li *et al* (58), detected a cancer risk reduction in *MSH6* rs1042821 heterozygotes or variant allele carriers. It should be noted that these observations in variant allele carriers do not contradict our prior suggestion of risk increase in variant homozygotes: considering, as stated above, i) the dual role of MSH6 on DNA repair and apoptosis; ii) the likely involvement of the MSH6 NTR in the differential regulation of such functions; and iii) the location and potential impact of rs1042821, it is possible that this SNP has distinct effects on each of MSH6 functions (DNA repair or apoptosis signalling) critically impairing one but somehow favouring the other. If so, variant allele homozygotes-lacking the common form of MSH6-could have higher cancer risk, while the presence of both forms in heterozygotes could be of some benefit. Further studies are required to confirm this hypothesis. Furthermore, it is interesting to note that two out of the three SNP pairs significantly associated with DTC susceptibility in our study involve variants that are located in the exact same chromosomic region of previously GWAS-suggested DTC markers. According to our *in silico* analysis, no linkage disequilibrium was identified between these co-localized variants in European populations. However, since it was not possible to verify this hypothesis with experimental data from our study, we cannot exclude the possibility that some of the variants analysed are indeed in linkage disequilibrium with previously suggested DTC markers, in the Portuguese population.

In conclusion, the rapidly increasing incidence of DTC (1) has prompted research on the genetic predisposing factors of this disease. Recently performed GWAS (6-12) have provided valuable contribution but, even so, explain only part of the estimated heritability of DTC (11,15,16). Several reasons may contribute: it is possible that the highly stringent criteria applied to GWAS to prevent false-positive findings result in the exclusion of SNPs truly associated with DTC risk (14). Furthermore, evidence from the latest GWAS (10-13) suggests the existence of population-specific DTC risk alleles, raising the possibility that novel cancer susceptibility markers, specific for geographically distinct populations, may remain to be identified. Finally, gene-gene and gene-environment interactions, despite seldom addressed, may also play an important role and explain part of the unresolved heritability of DTC susceptibility (15,16). The identification of additional common variants, gene-environment and gene-gene interactions predisposing to DTC may thus unveil at least part of the unexplained genetic component of DTC susceptibility. Hypothesis-driven case control association studies remain a valid approach and, as recently demonstrated (14), could provide valuable insight into the genetic risk factors for DTC.

This work suggests an involvement of MMR SNPs such as *MSH6* rs1042821, alone or in combination, on DTC susceptibility. However, despite the care put to avoid selection bias and variant misclassification, our results should be regarded solely as a proof of concept on the role of MMR genes on DTC susceptibility. Also, since the information that was collected from study participants on prior IR exposure was not suitable for rigorous statistical analysis, it was not possible to include it as a covariate in the adjustment statistical model. Since IR exposure remains the best-established risk factor for TC, future studies should be designed in order to account for this. Finally, since no SNP functional assessment was performed, we cannot exclude the possibility that the associations observed are due to other variants, in LD with the ones considered here. Therefore, in order to obtain conclusive evidence, these preliminary findings must be replicated in larger, multicentric studies with independent datasets of patients. Such studies should be powered to allow for more sophisticated analysis (e.g., haplotype analysis, evaluation of gene-gene and gene-environment interactions), for the study of other (e.g., less frequent but potentially relevant) variants and their potential association with mutational events that occur early in DTC carcinogenesis.

Acknowledgements

The authors acknowledge the invaluable contributions of their late colleague Professor Jorge Gaspar (1963-2015). The authors wish to thank Ana Paula Azevedo and Isabel Manita for technical support. This study was supported by funding through project UID/BIM/00009/2016 [Centre for Toxicogenomics and Human Health (ToxOmics)], from Fundação para a Ciência e a Tecnologia (FCT), Portugal.

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