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**Running Title: *MSH6* variants and familial breast cancer**

*Preclinical Study*

## **Association of rare *MSH6* variants with familial breast cancer**

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**Abstract**

Germline mutations in the mismatch repair genes *MLH1*, *MSH2*, *MSH6* and *PMS2* predispose to Lynch syndrome (also known as hereditary non-polyposis colorectal cancer). Recently, we have shown that the *CHEK2* 1100delC mutation also is associated with Lynch syndrome/Lynch syndrome-associated families albeit in a polygenic setting. Two of the ten *CHEK2* 1100delC positive Lynch syndrome families additionally carried a pathogenic *MLH1* or *MSH6* mutation, suggesting that mutations in mismatch repair genes may be involved in *CHEK2* 1100delC associated cancer phenotypes. A phenotype of importance is hereditary breast and colorectal cancer (HBC), with the *CHEK2* 1100delC mutation present in almost one-fifth of the families – again in a polygenic setting. To evaluate the involvement of *MSH6* in polygenic *CHEK2* cancer susceptibility, we here have analyzed the entire *MSH6* coding sequence for genetic alterations in 68 HBC breast cancer families. Rare *MSH6* variants, with population frequencies below 1%, were identified in 11.8% of HBC breast cancer families whereas the same variants were identified in only 1.5% of population controls, suggesting that rare *MSH6* variants are associated with HBC breast cancer ( $P < 0.00001$ ). However, screening of the entire *MSH6* coding sequence in 68 non-HBC breast cancer families showed a similar association (8.8% vs. ~1.4% in controls,  $P < 0.001$ ), suggesting that rare *MSH6* variants are not confined to HBC breast cancer. Together, our data suggest that rare *MSH6* variants may predispose to familial breast cancer. However, none of the rare *MSH6* variants are obviously pathogenic, suggesting that a more subtle disease mechanism may operate in breast carcinogenesis.

**Key words:** *CHEK2* 1100delC; Familial breast cancer; Hereditary breast and colorectal cancer; *MSH6*; Polygenic cancer susceptibility

## Introduction

In 2002, we and others have identified the truncating *CHEK2* 1100delC mutation as the first moderate-risk breast cancer susceptibility allele, present in about 5% of Dutch breast cancer families [1, 2]. In addition, we have shown that the *CHEK2* 1100delC mutation was also present among Lynch syndrome/Lynch syndrome-associated families and was particularly prevalent among breast cancer families with a hereditary breast and colorectal cancer (HBC) phenotype (4% and 18%, respectively) [3, 4]. However, in each of these instances, *CHEK2* 1100delC appeared to confer cancer risks in a polygenic setting.

The association of *CHEK2* 1100delC with colorectal cancer phenotypes suggested known colorectal cancer genes as likely candidates for polygenic *CHEK2* cancer susceptibility [4]. Germline mutations in the DNA mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6* and *PMS2* have been identified as the major causes for Lynch syndrome (previously also known as hereditary non-polyposis colorectal cancer, HNPCC; [5-7]). Interestingly, in two of the ten *CHEK2* 1100delC-positive Lynch syndrome-associated families we had also identified pathogenic mutations in *MHL1* or *MSH6* [4]. Concomitance of *CHEK2* and *MSH6* mutations had reportedly also been identified in a Finnish breast cancer family and in the colorectal cancer cell line HCT15 [8-11]. During repair of DNA damage, activated *CHEK2* is known to signal to *BRCA1*, which in turn acts as a scaffold protein for several DNA damage response proteins, including *MSH6* [12-14]. The close functional relation of *CHEK2* and *MSH6*, together with the observed concomitance of mutations in the two genes suggested that *MSH6* mutations may be involved in *CHEK2* polygenic cancer susceptibility. This hypothesis was particularly appealing for the HBC phenotype as *CHEK2* 1100delC is prevalent among these families whereas *MSH6* mutations are identified among Lynch syndrome families that also include breast cancer [3, 15-17]. Here, we therefore have screened the *MSH6* gene for genetic alterations in 68 HBC and 68 non-HBC breast cancer families.

## Patients and methods

### Breast cancer families and population controls

One hundred thirty-six breast cancer families were selected from 578 breast cancer families registered at the Rotterdam Family Cancer Clinic at Erasmus MC. Breast cancer families were classified as hereditary breast and colorectal cancer (HBCC) families (n=68) or non-HBCC families (n=68). All families included at least two first or second-degree relatives (DGRs) with breast cancer of whom at least one was diagnosed before the age of 60 years. HBCC families additionally included at least one patient with breast and colorectal cancer *or* at least one patient with colorectal cancer diagnosed before the age of 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 (3).

All breast cancer families were screened for germline mutations in the *BRCA1* and *BRCA2* genes and the *CHEK2* 1100delC mutation, identifying 26 *BRCA1* mutant families, 6 *BRCA2* mutant families and 18 families with the *CHEK2* 1100delC mutation. Two families were double mutant for *BRCA1* and the *CHEK2* 1100delC mutation. As part of our matching procedure, mutant *BRCA1*, *BRCA2* and *CHEK2* 1100delC families were equally divided over HBCC and non-HBCC families. Pathogenic germline mutations in the *MLH1* and *MSH2* gene were not identified in any of the 68 HBCC families. *MLH1* and *MSH2* mutation status had not been determined for the 68 non-HBCC families

All breast cancer families originated from the southwestern Netherlands and have consented to search for cancer susceptibility genes. The 166 control individuals were geographically-matched to the familial breast cancer cases and included spouses of heterozygous carriers of cystic fibrosis gene mutations, ascertained through the department of Clinical Genetics at Erasmus MC. The medical ethical committee of Erasmus MC approved this study.

### *MSH6* mutation analysis

The mismatch repair gene *MSH6* (NM\_000179.2) was screened for mutations in blood-derived DNA of the youngest diagnosed breast cancer case in the family (index case). The complete coding sequence of the *MSH6* gene, including intron/exon boundaries up to 25 bases in the intron, was amplified by

standard PCR. Amplified fragments were subsequently analyzed for genetic alterations by denaturing gradient gel electrophoresis (DGGE) or by direct sequencing as described [17-19]. Unique sequence alterations were confirmed at least once by sequence analysis of an independently generated PCR product. Primer sequences and reaction conditions are available upon request.

#### Statistical analysis

The difference between the mutation frequency in breast cancer patients versus controls was analysed using Fisher's Exact Test. *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

### Rare *MSH6* variants associate with HBCC breast cancer

We analyzed the entire *MSH6* coding sequence in 68 breast cancer families with hereditary breast and colorectal cancer (HBCC). Sequence analysis identified 25 different sequence alterations among 68 HBCC families, including 10 intronic and 15 exonic variants (Table 1). To evaluate the significance of the identified *MSH6* variants for HBCC, all variants were subsequently genotyped in 166 geographically-matched controls. Rare variants, with population frequencies below 1%, represented 56% (14/25) of the *MSH6* variants and each of these were identified only once or twice among the HBCC cohort. Four of the fourteen rare *MSH6* variants identified among HBCC families were also identified in the control cohort. Thus, although none of the rare *MSH6* variants associated significantly with HBCC families, the combined frequency of the rare variants was significantly higher among HBCC families compared to controls (16/136 (11.8%) vs. 5/306-332 (~1.5%),  $P < 0.00001$ ; Table 1). These results suggested that rare *MSH6* variants may predispose for HBCC breast cancer.

### Rare *MSH6* variants associate with familial breast cancer

To evaluate if rare *MSH6* variants associate with HBCC breast cancer or rather with familial breast

cancer in general, we also screened the entire *MSH6* coding sequence in 68 matched non-HBCC families (Table 1). Non-HBCC families were matched to the HBCC families with respect to their cancer pattern, i.e. the number of cancer patients per family, the number of cancer patients overall and the number of patients with single, double, triple or more cancers (Table 2). To avoid selection for HBCC, the non-HBCC families have been counter-selected for the presence of colorectal cancer. Six additional rare *MSH6* variants were identified among the non-HBCC families of which only one was also identified among the controls (Table 1). The combined frequency of rare *MSH6* variants was also significantly higher among non-HBCC families compared to controls (12/136 (8.8%) vs. 4/254-332 (~1.4%),  $P < 0.001$ ; Table 1). Hence, the prevalence of rare *MSH6* variants among non-HBCC families is equally high as among HBCC families, indicating that rare *MSH6* variants are not only associated with HBCC breast cancer but more likely with breast cancer in general. Combining the data on HBCC and non-HBCC families revealed a prevalence of rare *MSH6* variants of 10.3% among all breast cancer families whereas the same variants were identified in approximately 2.1% of controls ( $P < 0.0001$ ; Table 1), strongly suggesting that rare *MSH6* variants may predispose for familial breast cancer.

Do rare *MSH6* variants predispose for familial breast cancer?

Two aspects may question whether rare *MSH6* variants indeed predispose for familial breast cancer. First, we have not screened the entire *MSH6* coding sequence in the control cohort. Our strategy had been to genotype in the controls only those *MSH6* variants that had been identified among the familial breast cancer cohorts. Apart from the genotyped variant sequence, our primer design allowed analysis of the sequence surrounding the genotyped variant often including entire exon sequences and intron sequences up to 25 base pairs. In this way we were able to analyse 75% of the *MSH6* coding sequence in all controls. In addition to the five rare *MSH6* variants that already had been identified in the breast cancer families, two variants (c.59C>T, p.A20V and c.4002-10delCT) were once identified exclusively among the controls. Based on this low prevalence of rare *MSH6* variants that are exclusively present among the controls (2/254 (0.8%); Table 1), it may be anticipated that screening of the remaining quarter of the *MSH6* coding sequence in the controls is unlikely to identify many more rare *MSH6* variants.

Another aspect is the underlying disease mechanism of how rare *MSH6* variants may predispose to breast cancer. We found that the prevalence of rare *MSH6* variants was consistently higher among the breast cancer families than the controls, whether they were intronic or exonic and whether they were synonymous or non-synonymous. However, none of the identified rare *MSH6* variants are known pathogenic mutations. Evaluation of each of the rare *MSH6* variants with the splice prediction programs Berkeley Drosophila Genome Project ([www.fruitfly.org/seq\\_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html)) and NetGene2 ([www.cbs.dtu.dk/services/NetGene2/](http://www.cbs.dtu.dk/services/NetGene2/)) did not predict structural effects. It thus remains unclear how these rare *MSH6* variants exert their putative oncogenic effect, particularly for the intronic variants and the non-synonymous exonic variants. But then, are obvious pathogenic effects to be expected in a polygenic setting? Importantly, the statistical evidence from our analysis that rare *MSH6* variants associate with familial breast cancer is compelling ( $P < 0.001$ ), strongly suggesting cancer predisposition by rare *MSH6* variants. Obviously, our analysis requires replication in independent familial breast cancer cohorts, and it seems also warranted to screen familial colorectal cancer cohorts, with particular focus to rare variants that are not obviously pathogenic. In this respect, our current findings are consistent with a report by Nevanlinna and colleagues in which 15 different *MSH6* variants were identified among 38 breast cancer families with colorectal cancer and/or endometrial cancer [20]. Three of the *MSH6* variants classified as rare variants, including two synonymous exonic variants and one intronic variant. The prevalence of rare *MSH6* variants in their familial breast cancer cohort is similar to the prevalence we report here ( $3/38=7.9\%$  versus  $10.3\%$  in our cohort) and supports our conclusion that rare *MSH6* variants are associated with familial breast cancer. Their and our observations both seem to point towards a currently unknown disease mechanism in breast carcinogenesis such as modulation of transcript expression levels or mediation of non-coding RNAs located in the genomic regions associated with these variants. Therefore, one may wonder whether this mechanism is similar to the as-yet unresolved disease mechanism underlying the more prevalent low-risk breast cancer alleles that recently have received much attention [21-26].

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## Legends to the tables

**Table 1.** *MSH6* mutation analysis among 68 HBCC and 68 non-HBCC breast cancer families

<sup>a</sup> Numbering of nucleotide changes according *MSH6* GenBank sequence NM\_000179.2

<sup>b</sup> Frame shift mutation is indicated by the first changed codon and the number of newly encoded codons, including premature termination codon X. This particular variant locates 3' in the gene sequence predicting a protein shortened with only two amino acids.

BRC, breast cancer; HBCC, hereditary breast and colorectal cancer.

**Table 2.** Cancer incidence among 68 HBCC and 68 non-HBCC breast cancer families

BRC, breast cancer; CRC, colorectal cancer; HBCC, hereditary breast and colorectal cancer families;

other cancer cases, cancer cases other than breast cancer or colorectal cancer.

MSH6 gene sequence <sup>a</sup>	Predicted protein effect	Minor allele / Total tested alleles (%)								
		HBCC		Non-HBCC		All BRC		Controls		
<b>Prevalent variants (&gt;1%)</b>										
-	-	31/136	(22.8)	23/136	(16.9)	54/272	(19.9)	58/332	(17.5)	
<b>c.260+22C&gt;G</b>										
c.3438+14A>T	-	58/136	(42.6)	58/136	(42.6)	116/272	(42.6)	126/322	(39.1)	
c.3557-4dupT	-	26/136	(19.1)	26/136	(19.1)	52/272	(19.1)	56/324	(17.3)	
c.4002-10delT	-	35/136	(25.7)	39/136	(28.7)	74/272	(27.2)	2/328	(25.6)	
c.4002-10dupT	-	14/136	(10.3)	16/136	(11.8)	30/272	(11.0)	41/312	(13.1)	
c.116G>A	p.G39E	25/136	(18.4)	24/136	(17.6)	49/272	(18.0)	52/332	(15.7)	
c.186C>A	p.=	31/136	(22.8)	23/136	(16.9)	54/272	(19.9)	58/332	(17.5)	
c.276A>G	p.=	29/136	(21.3)	22/136	(16.2)	51/272	(18.9)	53/332	(16.0)	
c.540T>C	p.=	42/136	(30.9)	39/136	(28.7)	81/272	(29.8)	95/332	(28.6)	
c.642C>T	p.=	12/136	(8.8)	14/136	(10.3)	26/272	(9.6)	38/332	(11.4)	
c.1286C>G	p.L396V	2/136	(1.5)	0/136	-	2/272	(0.7)	6/332	(1.8)	
<b>Rare variants (&lt;1%)</b>										
<i>Intronic (up to 25 bases)</i>										
-	-	1/136	(0.7)	0/136	-	1/272	(0.4)	1/332	(0.3)	
<b>c.457+13A&gt;G</b>										
c.3439-16C>T	-	2/136	(1.5)	0/136	-	2/272	(0.7)	0/306	-	
c.3647-11T>G	-	0/136	-	1/136	(0.7)	1/272	(0.4)	0/254	-	
c.4001+12_4001+15delACTA	-	2/136	(1.5)	1/136	(0.7)	3/272	(1.1)	2/328	(0.6)	
c.4002-10delTT	-	1/136	(0.7)	0/136	-	1/272	(0.4)	0/312	-	
c.4002-10delTTT	-	0/136	-	1/136	(0.7)	1/272	(0.4)	1/312	(0.3)	
c.4002-10delTTTT	-	0/136	-	1/136	(0.7)	1/272	(0.4)	0/312	-	
c.4002-10dupTTT	-	1/136	(0.7)	1/136	(0.7)	2/272	(0.7)	0/312	-	
<i>Exonic synonymous</i>										
c.1053C>T	p.=	1/136	(0.7)	0/136	-	1/272	(0.4)	0/332	-	
c.1164C>T	p.=	0/136	-	1/136	(0.7)	1/272	(0.4)	0/332	-	
c.2272C>T	p.=	0/136	-	1/136	(0.7)	1/272	(0.4)	0/332	-	
c.2775A>C	p.=	1/136	(0.7)	0/136	-	1/272	(0.4)	0/332	-	
c.3246G>T	p.=	1/136	(0.7)	2/136	(1.5)	3/272	(1.1)	0/332	-	
c.3306T>A	p.=	1/136	(0.7)	1/136	(0.7)	2/272	(0.7)	0/332	-	
<i>Exonic non-synonymous</i>										
c.73G>T	p.A25S	1/136	(0.7)	1/136	(0.7)	2/272	(0.7)	1/332	(0.3)	
c.751A>G	p.I251V	1/136	(0.7)	0/136	-	1/272	(0.4)	0/332	-	
c.1508C>G	p.S503C	1/136	(0.7)	0/136	-	1/272	(0.4)	1/332	(0.3)	
c.2045C>T	p.S682F	1/136	(0.7)	0/136	-	1/272	(0.4)	0/332	-	
c.2633T>C	p.V878A	1/136	(0.7)	0/136	-	1/272	(0.4)	0/332	-	
c.4072_4075dupGATT	p.K1358fsX2 <sup>b</sup>	0/136	-	1/136	(0.7)	1/272	(0.4)	0/332	-	
<b>Summary rare variants</b>										
		Minor allele / Total tested alleles (%)								
	All	Intronic		Exonic all		Exonic synonymous		Exonic non-synonymous		
HBCC families	16/136	(11.8)	7/136	(5.1)	9/136	(6.6)	4/136	(2.9)	5/136	(3.7)
Controls	5/306-332	(~1.5)	3/306-332	(~0.9)	2/332	(0.6)	0/332	(~0.0)	2/332-332	(0.6)
P-value	<0.00001									
Non-HBCC families	12/136	(8.8)	5/136	(3.7)	7/136	(5.1)	5/136	(3.7)	2/136	(1.5)
Controls	4/254-332	(~1.4)	3/254-328	(~1.1)	1/332	(0.3)	0/332	(~0.0)	1/332	(0.3)
P-value	<0.001									
All BRC families	28/272	(10.3)	12/272	(4.4)	16/272	(5.9)	9/272	(3.3)	7/272	(2.6)
Controls	6/254-332	(~2.1)	4/254-332	(~1.4)	2/332	(0.6)	0/332	(~0.0)	2/332	(0.6)
P-value	<0.0001									

