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Very low prevalence of germline MSH6 mutations in hereditary non-polyposis colorectal cancer suspected patients with colorectal cancer without microsatellite instability

Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal-dominant inherited disorder predisposing to colorectal cancer (CRC) and several other cancers at an early age, including endometrial carcinoma. It is clinically suspected by Amsterdam criteria and Bethesda guidelines (Rodriguez-Bigas et al., 1997; Umar et al., 2004). Hereditary non-polyposis colorectal cancer is caused by mutations in one of the mismatch repair (MMR) genes (MLH1, MSH2, MSH6, and PMS2) and is characterised by tumours that show microsatellite instability (MSI). Failure of MMR results in MSI especially in short repetitive sequences. Molecular testing for HNPCC can be performed by testing tumours for MSI-high and absence of MLH1, PMS2, MSH2 and/or MSH6 as determined by immunohistochemistry (IHC), and germline mutation analysis of MMR genes.

In clinical practice MSI analysis is used as a prescreening tool to detect colorectal cancer without an MSI-high pattern (Berends et al., 2002; Hendriks et al., 2004; Plaschke et al., 2004), whereas in MLH1 and MSH2 mutation carriers almost all HNPCC-associated tumours show MSI (Lynch and Lynch, 2005). The reliability of MSI analysis to select patients at risk for MSH6 mutations is therefore questioned. As germline mutation analysis and IHC of MMR proteins is almost exclusively initiated when MSI analysis shows MSI, we might miss MSH6 germline mutations.

The aim of this study was to establish the prevalence of MSH6 mutations in HNPCC suspected patients without MSI in their tumours to investigate the value of MSI analysis to detect MSH6 mutations.

**MATERIALS AND METHODS**

The study is based on 617 tumours of patients or their family members suspected of HNPCC that visited our clinical genetics department in which MSI and subsequent analyses were performed between 1997 until 2006 (Figure 1). In the families analysed in our study MSI analysis is performed in the tumour of the youngest relative available. All findings in this group that were available at 1-1-2006 are included in this study. In 529 tumours of patients a reliable distinction between MSI-high and MSI-stable/low could be made using the standard set of markers (Boland et al., 1998). IHC of MMR proteins became available and was applied for from 1999,
in some cases retrospectively, IHC of the MSH6 protein was performed in all tumours regardless of MSI results. IHC of all MMR proteins was performed in case of an MSI-high or MSI-low tumour or when other tissue than CRC was tested, such as endometrial cancer, gastric cancer, sebaceous carcinoma, urothelial cell carcinoma, and brain tumours (Rodriguez-Bigas et al., 1997). We focused on two separate cohorts of patients; patients with and without MSI in their tumour DNA. The pedigrees made as a part of the genetic counselling procedure were studied for fulfilment of Amsterdam II criteria and Bethesda guidelines (Rodriguez-Bigas et al., 1997; Umar et al., 2004).

The study was performed according to the rules of the Medical Ethics Committee of the Radboud University Nijmegen Medical Centre.

Molecular analysis

For MSI analysis normal and tumour tissues were extracted from formalin-fixed and paraffin-embedded tissues. The Bethesda microsatellite panel D2S123, D5S346, D17S250, BAT25, and BAT26 (Boland et al., 1998) was used essentially according to methods described previously (Hoogerbrugge et al., 2003). A tumour was considered MSI-high when instability was found in ≥2 out of five markers (n = 91) and MSI-stable or low in case of instability in ≤1 out of five markers (n = 438). In 178 samples the mononucleotide marker BAT40 was included in the standard marker set. IHC of the MMR proteins was performed with the monoclonal antibodies against MSH6 (Transduction lab code: G70220), MLH1 (Pharmingen code: 51–1327/GR), PMS2 (Pharmingen code: 556415), and MSH2 (Oncogene code: NA26). Germline MSH6 mutation analysis of the coding regions and splice sites of the MSH6 gene was performed with a combination of sequence analysis (exon 1, splice acceptor site of exon 10), one-dimensional denaturing gradient gel electrophoresis (exons 2 up to and including 10) essentially as described by Wu et al. (1999) and multiplex ligation-dependent probe amplification (MRC Holland) for the detection of exon deletions and duplications (exon 1 to 10). Only changes located within 10 nucleotides of the coding region that have not been described as polymorphisms before, are reported.

Patients with an MSI-high tumour

MSH6 germline mutation analysis was performed in a group of 19 patients with MSI-high HNPCC-associated tumours and loss of MSH6 expression in which MLH1 and MSH2 mutations were excluded. Nine of these tumours showed loss of MSH6 expression in the presence of MSH2 expression and 10 showed loss of both MSH2 and MSH6 expression, of which two were difficult to interpret and possibly also showed loss of PMS2 expression. Microsatellite instability patterns of HNPCC-associated tumours of 12 MLH1, 22 MSH2, and 10 MSH6 mutation carriers were studied to compare the instability patterns of tumours of patients with
germline mutations in MSH6 to those with germline mutations in MLH1 and MSH2.

Patients with a non-MSI-high tumour

Three hundred and sixty-three non-MSI-high HNPCC-associated tumours (295 CRC) were analysed out of 335 families. Patients most suspected of HNPCC were selected by fulfilment of at least one of the following criteria: (1) age at diagnosis below 50 years, (2) first degree relative with an HNPCC-related tumour, or (3) second CRC. Of the patients that fulfilled one or more of these criteria a subgroup of 89 patients, 76 of whom had CRC, and one first degree relative, were analysed for MSH6 germline mutations.

Statistical analysis

Categorical variables were compared with the use of the Fisher’s exact test using SPSS, version 12.0. A P-value of 0.05 is considered as threshold for statistical significance.

RESULTS

MSH6 mutation analysis in patients with an MSI-high tumour

In a group of 19 patients with both an MSI-high HNPCC-associated tumour and loss of MSH6 expression, but no detectable defect in MLH1 or MSH2, 10 pathogenic mutations in MSH6 were found in nine families (Table 1). Besides the nine different MSH6 germline mutations found in patients with an MSI-high tumour, two pathogenic mutations in MSH6 were found in patients in whom MSI analysis could not be performed. The mean age at diagnosis of the 11 index patients from the families with a pathogenic MSH6 mutation was 44 years (range 36 – 57). The MSH6 analyses in nine of these index patients with an MSH6 mutation was performed on four endometrial, four colorectal, and one urothelial cell cancer. All MSH6 mutation carriers fulfill one or more Bethesda guidelines and in 64% of the families the Amsterdam II criteria are fulfilled. In the MSH6 families endometrial cancers occur as frequently as CRCs.

Of the remaining nine tumours with loss of MSH6 expression, eight tumours also showed loss of MSH2 expression of which two were difficult to interpret and possibly showed loss of PMS2 expression as well, suggesting the presence of an as yet undetected MSH2 (or PMS2) germline mutation. One tumour, a CRC developed at age 53, exclusively showed loss of the MSH6 protein. In this female patient an MSH6 variant c.2117T > C (p.Phe706Ser) was found of which the pathogenicity is uncertain. She also carries a pathogenic mutation in BRCA2 (c.3269del (p.Met1086fs)). The patients’ mother carries the same MSH6 variant but not the BRCA2 mutation. She was diagnosed with endometrial cancer at age 62. Microsatellite instability analysis and IHC on her tumour were inconclusive.

Stability in one or more of the dinucleotide markers occurred significantly more often in colorectal tumours of MSH6 than of MLH1 and MSH2 mutation carriers (Table 2). Stability of

Table 1: Characteristics of patients with a germline mutation in MSH6

| Tested cancer and age at diagnosis | Pathogenic mutation MSH6 | Instable mono-nucleotides | Stable di-nucleotides | IHC MSH6 | IHC MSH2 | Amsterdam criteria II | Bethesda A | Bethesda B | EN in family | CRC in family |
|-----------------------------------|--------------------------|--------------------------|----------------------|---------|---------|----------------------|----------|---------|-------------|--------------|
| CO 42                            | c.265_–1_457+1dup        | 2/3                      | 2/3                  | Neg     | Pos     | +                    | +        | +       | +           | +            |
| EN 57a                           | c.314G>T (p.Glu722X)     | 3/3                      | 0/3                  | Neg     | Pos     | –                    | +        | –       | –           | –            |
| CO 52c                          | c.651dup (p.Lys218X)     | 2/2                      | 3/3                  | Neg     | Pos     | +                    | EN 37    | +       | +           | +            |
| EN 58a                           | c.651dup (p.Lys218X)     | 3/3                      | 2/3                  | Neg     | Pos     | +                    | +        | +       | +           | +            |
| EN 56                            | c.3237dup (p.Lys1092X)   | 2/2                      | 2/3                  | Neg     | Pos     | +                    | +        | +       | +           | +            |
| EN 43                            | c.3261dup (p.Phe1086I)   | 2/2                      | 3/3                  | Neg     | Neg     | –                    | –        | –       | –           | –            |
| CO 39                            | c.3261del (p.Phe1086I)   | 2/2                      | 2/3                  | Neg     | Pos     | –                    | –        | –       | –           | –            |
| EN 38                            | c.1135_1139del (p.Arg379X) | 2/2                     | 1/3                  | Neg     | Pos     | –                    | +        | –       | –           | –            |
| UR 56                            | c.1_475+1del             | 3/3                      | 0/3                  | Neg     | Pos     | –                    | +       | +       | –           | –            |
| EN 38                            | c.3678_306dup (p.Ala1236fs) | nt                      | nt                   | nt      | nt      | –                    | –        | +       | –           | –            |
| CO 47                            | c.2815C>T (p.Gln939X)    | nt                      | nt                   | nt      | nt      | –                    | –        | +       | –           | –            |
| Total                            |                          |                         |                      |         |         |                     |          |         |             |              |

Bethesda A: Proband with two HNPCC-related cancers, Bethesda B: Proband and first degree relative with HNPCC-related cancer, one diagnosed <50 y. EN = endometrial cancer, CO = colorectal cancer, UR = urothelial cell carcinoma, SEB = sebaceous adenoma, O = ovarian cancer; Neg = negative, Pos = positive, nt = not tested; IHC = immunohistochemistry; MSH = microsatellite instability. *This patient also has an UV c.65G>C (p.Gly22Ala) in MLH1. Patients from same family. **Tumour of patients father showed MSI and no MSH6 expression.

Table 2: Results of the MSI analysis in MSH6, MLH1 and MSH2 mutation carriers

| MSI pattern | MSH6 mutation carriers | MLH1 and MSH2 mutation carriers | P-value Fisher exact |
|-------------|------------------------|---------------------------------|---------------------|
| One or more of three dinucleotides* stable | CRC 4/5 (80%) | 4/22 (18%) | **0.017** |
| Non CRC    | 4/5 (80%)             | 1/6 (17%) | NS     |
| One or more mononucleotides* stable    | CRC only 1/5 (20%) | 2/26 (8%) | NS     |
| Non CRC    | 0/5 (0%)              | 0/6 (0%)  | NS     |

NS = not significant; CRC = colorectal cancer; MSH = microsatellite instability. *D2S123, D5S346, and D17S250. **BAT25 and BAT26.
mononucleotide markers is uncommon in tumours of MSH6 as well as MLH1 and MSH2 mutation carriers.

**MSH6 mutation analysis in patients with a non-MSI-high tumour**

Immunohistochemical staining showed MSH6 expression in all 295 non-MSI-high CRCs and in 67 out of 68 other non-MSI-high HNPCC-related tumours (Table 3).

A subgroup of patients with the highest suspicion of HNPCC, was tested for the presence of MSH6 germline mutations. In none of the 76 patients with CRC, or in the 13 patients with other HNPCC-related tumours a pathogenic germline mutation in MSH6 was detected. One non-MSI-high tumour of metastatic tumour tissue (most probably derived from a CRC) of a deceased patient was detected. One non-MSI-high tumour of metastatic tissue (most probably derived from a CRC) of a deceased patient showed loss of MSH6 expression, in presence of MLH1 and MSH2 expression. Because mutation analysis could not be performed in the deceased patient, mutation analysis in her brother was performed. No mutation in MSH6 was detected (Table 4).

Silent variants c.3852G>A, c.2154C>T, c.1068T>C, and c.3246G>T were found. None of these are predicted to affect splicing and thus do not seem to have functional consequences. The missense variant c.3101G>C (p.Arg1034Pro) that was found in a female patient with CRC at age 43 might be pathogenic. As the carcinoma was not available the MSI and IHC analyses were performed in an adenoma, which might have decreased the sensitivity of the analyses. Segregation analysis in the family showed that her mother and her brother, the mother who had two sisters with anamnestic endometrial cancer did not carry the MSH6 variant, making the pathogenicity of this variant less likely.

**DISCUSSION**

In this study, not one pathogenic germline MSH6 mutation was detected in HNPCC suspected patients with a non-MSI-high CRC or HNPCC-related tumour.

Previous studies suggested that the sensitivity of MSI analysis to predict an MSH6 mutation is low and that MSI should not be used as a selection criterion for MSH6 mutation analysis (Wu et al, 1999), finding microsatellite stable or low patterns in 17% up to 50% (Berends et al, 2002; Hendriks et al, 2004; Plaschke et al, 2004; Niessen et al, 2006; Pinto et al, 2006) of HNPCC-associated tumours of MSH6 mutation carriers. However careful consideration of previous studies is required as part of the conclusions are based on MSH6 missense mutations of unknown pathogenecity or testing a sporadic tumour within an HNPCC family (a phenocopy) as suggested by positive immunostaining of MSH6 in the tumour. These show an unfavourable effect on the sensitivity of MSI analysis. In addition MSI analysis on endometrial cancer, the most frequent tumour in female MSH6 mutation carriers might decrease its sensitivity, as it is known that the instability in these tumours is generally less pronounced (Wijnen et al, 1999; Hendriks et al, 2004).

MSH6 mutations result in a weaker mutator phenotype (Kolodner et al, 1999), which may be explained by the major function of MSH6 to correct base–base mismatches and single nucleotide deletion loops but not larger deletion loops (Parc et al, 2000). Like in previous studies (Kolodner et al, 1999; Verma et al, 1999; Parc et al, 2000) our study shows that mononucleotide markers but not dinucleotide markers are sensitive to show instability in tumours of MSH6 mutation carriers. The sensitivity of MSI analysis therefore depends on the microsatellite markers used. Enlarging the standard (Bethesda) marker set (Boland et al, 1998) with a mononucleotide marker (like BAT40) will increase the sensitivity of MSI analysis by minimising the chance of missing tumours with MSH6 inactivation. As data on MSI analysis of other non-colorectal HNPCC-related tumours with defective MMR are insufficient, we recommend additional IHC of MLH1, PMS2, MSH2, and MSH6 proteins when MSI analysis is performed on non-colorectal HNPCC-related cancers. Immunohistochemical staining of MMR proteins will also improve the interpretation of MSI patterns when a low percentage of tumour cells or an adenoma is tested or when only one mononucleotide marker shows instability (MSI low). When a patient is excluded from further HNPCC analysis based on a non-MSI-high pattern in

### Table 3  Overview of microsatellite stable/low tumours

| Patient with non-MSI-high tumour and loss of MSH6 expression | Selected group of patients with non-MSI-high tumours and positive MSH6 expression | Patients with non-MSI-high tumours and positive MSH6 expression | Patients with non-MSI-high tumours and positive MSH6 expression |
|------------------------------------------------------------|---------------------------------------------------------------------------|------------------------------------------------------------|------------------------------------------------------------|
| Colorectal ca                                              |                                                                           | 295 (58%)                                                 | 171 (58%)                                                 |
| Age < 50 yr                                                |                                                                           |                                                           | 62 (82%)                                                   |
| Other HNPCC-related neoplasia                             |                                                                           | 1                                                         | 67                                                        |
| Endometrial ca                                             |                                                                           | 15                                                        | 3                                                         |
| Gastric ca                                                 |                                                                           | 3                                                         |                                                           |
| Sebaceous ca                                               |                                                                           | 4                                                         |                                                           |
| Urothelial cell ca                                         |                                                                           | 1                                                         |                                                           |
| Brain tumour                                               |                                                                           | 1                                                         |                                                           |
| Metastatic tissue                                          | 1*                                                                       | 7                                                         |                                                           |
| Small bowel                                                |                                                                           | 1                                                         |                                                           |
| Adenoma                                                    |                                                                           | 34                                                        | 10                                                        |
| Colon                                                      |                                                                           |                                                           |                                                           |
| Duodenum                                                   |                                                                           |                                                           |                                                           |
| Age < 50 yr                                                | 0                                                                        | 34 (51%)                                                  | 9 (69%)                                                   |

MSI = microsatellite instability; HNPCC = hereditary non-polyposis colorectal cancer; ca = cancer. *Mutation analysis in the patients’ brother showed no MSH6 mutation.

### Table 4  MSI-test result and IHC protein expression pattern of tumours from patients tested for the presence of a MSH6 germline mutation

| MSI         | MSI high | MSI stable/low |
|-------------|----------|----------------|
| IHC         | MSH6+    | MSH6-          |
|             | MSH2+    | MSH2-          |
|             | MLH1+    | MLH1-          |
|             | PMS2+    | PMS2-          |
| No pathogenic mutation in MSH6 | 1* | 6 |
| Pathogenic mutation in MSH6 | 8 |

IHC = immunohistochemistry; MSI = microsatellite instability. *IHC difficult to interpret. **With MSH6 variant c.3117T>C (p.Phe706Ser). *Mutation analysis in the patients’ brother.
tumour DNA, a second MSI analysis in the family should always be considered to avoid missing a germline mutation because of an initial test in a pheno-coppy.

From previous studies we know that, in MSH6 mutation carriers CRC occurs at older age than in MLH1 and MSH2 mutation carriers (Hendriks et al, 2004). In our study, the patients with MSI-stable/low tumours that were analysed for MSH6 mutations were mainly diagnosed before the age of 50. This selection is not expected to have a large influence, because MSI analysis in the families in our study is performed in the tumour of the youngest relative available. The mean age of diagnosis in MSH6 mutation carriers is above 50, but the occurrence of one relative below 50 is expected to be present in most of the MSH6 families. The pedigrees of the diagnosed MSH6 families in our study all contained an affected relative diagnosed below 50 years of age.

The prevalence of MSH6 mutation carriers in HNPPC suspected CRC patients is low, as is demonstrated by the fact that we detected an MSH6 mutation in only about 1% of these patients. All these mutations were found in patients with an MSI-high tumour. Data from previous studies (Berends et al, 2002; Hendriks et al, 2004; Barnetson et al, 2006; Niessen et al, 2006) show that approximately 15% of colorectal tumours of MSH6 mutation carriers do not have an MSI-high pattern, whereas they do show loss of MSH6 expression and thus might be the result of the MSH6 germline mutation. On the other hand, approximately 5% of colorectal tumours of MSH6 mutation carriers do show neither an MSI-high pattern nor loss of MSH6 expression and thus might have arisen independent from the genetic background of the carrier. Based on our finding of the low incidence of MSH6 mutations in HNPPC-suspected CRC patients and the percentage of non-MSI-high tumours in MSH6 mutation carriers from the literature, the probability of missing a mutation by not performing mutation analyses in patients with non-MSI-high CRCs is expected to be extremely low. This is confirmed by the fact that we did not find any non-MSI-high CRC with loss of MSH6 expression, nor a germline MSH6 mutation in any of the patients with a non-MSI-high tumour. Our findings show that MSI analysis is highly suited to trace CRC of carriers of MSH6 germline mutations.

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