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Genomic rearrangements in *MSH2*, *MLH1* or *MSH6* are rare in HNPCC patients carrying point mutations

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Abstract

Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominant disease with high penetrance, caused by germline mutations in the mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6*, *PMS2* and *MLH3*. Most reported pathogenic mutations are point mutations, comprising single base substitutions, small insertions and deletions. In addition, genomic rearrangements, such as large deletions and duplications not detectable by PCR and Sanger sequencing, have been identified in a significant proportion of HNPCC families, which do not carry a pathogenic MMR gene point mutation.

To clarify whether genomic rearrangements in *MLH1*, *MSH2* or *MSH6* also occur in patients carrying a point mutation, we subjected normal tissue DNA of 137 colorectal cancer (CRC) patients to multiplex ligation-dependent probe amplification (MLPA) analysis. Patients fulfilled the following pre-requisites: all patients met at least one criterion of the Bethesda guidelines and their tumors exhibited high microsatellite instability (MSI-H) and/or showed loss of expression of MLH1, MSH2 or MSH6 proteins. PCR amplification and Sanger sequencing of all exons of at least one MMR gene, whose protein expression had been lost in the tumor tissue, identified 52 index patients without a point mutation (Group 1), 71 index patients with a pathogenic point mutation in *MLH1* ($n = 38$) or *MSH2* ($n = 22$) or *MSH6* ($n = 11$) (Group 2) and 14 patients with an unclassified variant in *MLH1* ($n = 9$) or *MSH2* ($n = 3$) or *MSH6* ($n = 2$) (Group 3). In 13 of 52 patients of group 1 deletions of at least one exon were identified. In addition, in group 3 one EX1_15del in *MLH1* was found. No genomic rearrangement was identified in group 2 patients.

Genomic rearrangements represent a significant proportion of pathogenic mutations of MMR genes in HNPCC patients. However, genomic rearrangements are rare in patients carrying point mutations in MMR genes. These findings suggest the use of genomic rearrangement tests in addition to Sanger sequencing in HNPCC patients.

Keywords: HNPCC; Exon deletions; Mismatch repair genes

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1. Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC) is caused by germline mutations in the mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6*, *PMS2* or*MLH3* [1–11]. Most reported pathogenic mutations are point mutations, comprising single base substitutions, small insertions and deletions [12,13]. In addition, genomic rearrangements such as large deletions and duplications, which cannot be detected by PCR and Sanger sequencing analysis, have been identified in a significant proportion of HNPCC families [14–23]. Yet, in most studies only patients in which no pathogenic point mutation had been found were screened for large genomic rearrangements in MMR genes.

Although several cases with biallelic mutations in MMR genes [24–35] have been published, there is no report on compound heterozygous mutations comprising point mutations and genomic rearrangements. This may be due to screening for genomic rearrangements not being a standard procedure until recently. Yet, biallelic mutations in MMR genes have significant clinical consequences and are important for predictive molecular testing in the respective family.

To clarify whether both point mutations and genomic rearrangements in *MLH*, *MSH2* or *MSH6* are the cause of disease in single individuals, we performed a study in 137 unrelated HNPCC patients.

2. Materials and methods

2.1. Patients

All index patients met at least one criterion of the Bethesda guidelines [36] and their colorectal cancer tissue exhibited high microsatellite instability (MSI-H) and/or showed loss of expression of MLH1, MSH2 or MSH6 proteins. All patients had been subjected to DNA-sequencing of all exons and adjacent intronic regions of at least one MMR gene whose protein expression had been lost in the tumor tissue. We analysed all patients for large genomic rearrangements in *MLH1*, *MSH2* and *MSH6* using the multiplex ligation-dependent probe amplification (MLPA) analysis. There were 52 index patients (11 (21.2%) of them fulfilling the Amsterdam-criteria) without pathogenic point mutations in MMR genes (Group 1), 71 index patients (29 (40.8%) of them fulfilling the Amsterdam-criteria) with a pathogenic point mutation in *MLH1* ($n = 38$), *MSH2* ($n = 22$) and *MSH6* ($n = 11$) (Group 2) and 14 index patients (6 (42.9%) of them fulfilling the Amsterdam-criteria) with an unclassified variant (UV, i.e. missense mutation) in *MLH1* ($n = 9$), *MSH2* ($n = 3$) and *MSH6* ($n = 2$) (Group 3).

2.2. Screening for rearrangements by multiplex ligation-dependent probe assay (MLPA)

MLPA was perfomed as described by Schouten et al. [37] on genomic DNA isolated from peripheral blood leukocytes. Genomic rearrangements in *MLH1*, *MSH2* and*MSH6* genes were evaluated by MLPA test kits P003 for *MLH1*/*MSH2 and* P008 for*MSH6*/*PMS2* (MRC-Holland, Amsterdam, The Netherlands) and performed according to the supplied protocol. The test kit for *MLH1*/*MSH2* contains 16 exon probes for *MSH2*, 19 exon probes for *MLH1*, and 7 control probes specific for DNA sequences outside those genes. The test kit for *MSH6*/*PMS2* includes 10 exon probes for *MSH6*. Genomic DNA (20–500 ng) was denaturated in 5 μl Tris EDTA (TE) at 98 °C for 5 min and incubated with the probe mix for 16 h at 60 °C. Following probe hybridization, ligation proceeded for 15 min at 54 °C. Then, ligation products were amplified by polymerase chain reaction (PCR) using a FAM-labeled primer and an unlabeled primer. PCR products were analysed on ABI 3700 capillary sequencer using GeneMapper® software (Applied Biosystems, Forster City, CA, USA). Specific peaks corresponding to each exon were identified according to their migration in relation to size standards. Peak heights of each fragment were compared to those of control samples and deletions were suspected when peak height differed by more than 30% [23].

2.3. Breakpoint analysis and long range PCR

In patient 2430, breakpoint analysis and identification of the unknown upstream sequence was performed using the Universal Genome Walker™ Kit (Clontech Laboratories, Palo Alto, CA, USA) according to the manufacturers protocol. In brief, genomic DNA from patients and from a normal control was digested with *Eco*RV, *Dra*I, *Pvu*II and *Stu*I. DNA fragments were ligated with a Genome Walker adapter. PCR was performed with the sense primer AP1 supplied with the Genome Walker Kit and the specific antisense primer GSP1: 5′-GCACCCGGCT GGAAATTTTATTTG-3′ located in intron 16 of the MLH1 gene. Subsequently nested PCR was performed with the sense primer AP2 supplied with the Genome Walker Kit and the specific antisense primer GSP2: 5′-TTGAACCTGGAAGGCTGAGGTTA-3′ located in intron 15.

Amplified DNA fragments were analyzed by direct DNA-sequencing, with the thermo sequenase fluorescent cycle sequencing kit (Amersham Pharmacia Biotech, Freiburg, Germany). Sequencing primers were identical to PCR primers (AP2 and GSP2) with additional Cy5-labeling, allowing sequence analysis on ALFexpress devices (Amersham Pharmacia Biotech, Freiburg, Germany).

Denaturing 6.5% Long Ranger gels were used and prepared according to the manufacturer's instructions (FMC Bioproducts, Vallensbaek Strand, Denmark). Runs were analyzed by using ALF evaluation software (Amersham Pharmacia Biotech, Freiburg, Germany).

Long-range PCR on genomic DNA was used to confirm the deletion uncovered by multiplex PCR and to identify the allele harbouring the missense mutation in Exon 16 of patient 2430. Long-range PCR from exon 15 to exon 16 was performed using the Expand Long Template PCR system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. After purification/separation by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining, exon 16 was directly sequenced on an automated sequencer using Cycle Sequencing Kit (Amersham Pharmacia Biotech, Freiburg, Germany) and primer sequences as described elsewhere [3].

3. Results

Genomic deletions of one or more exons were detected in 13 of 52 patients in group 1 (three in *MLH1* and ten in *MSH2*), all predicted to be pathogenic (Table 1). Two patients fulfilled the Amsterdam-criteria. Two mutations (EX1_3del and EX1_16del of *MSH2*) were identified twice. In addition, we identified an EX1_15del in *MLH1* in patient 2430 of group 3 (Fig. 1).

The EX1_15del in *MLH1* in patient 2430 was verified by breakpoint analysis and long-range PCR. We identified a 105.565 bp deletion between two Alu repeats of the Sx type family (Fig. 2). Allele specific PCR comprising exons 15 and 16 and subsequent sequence analysis revealed the missense mutation c.1853A > G (p.K618R) to be located on the allele harbouring the EX1_15del.

4. Discussion

Genomic rearrangements represent a significant proportion of all pathogenic mutations in MMR genes in HNPCC patients. Several HNPCC study groups have shown that genomic rearrangements comprise 15% to 55% of all MMR mutations [14,16,17,20,22,38,39].

In our study, in 14 of 137 index patients suspected of HNPCC a pathogenic genomic deletion in *MLH1* or *MSH2* was identified, comprising 16.5% (14 of 85) of all pathogenic mutations identified in our patients. In contrast to the North American finding of a founder mutation EX1_6 in *MSH2* [40], we have identified two different deletions (EX1_3del and EX1_16del of *MSH2*) more than once. However, we and another German study group have also identified one patient with an EX1_6del [20,22,41].

We identified only one patient with two mutations, an EX1_15del of *MLH1* (Fig. 1), and a c.1853A > G transition, which results in p.K618R. Yet, allele specific PCR showed that both mutations are located on the same allele, which predicts that the genomic rearrangement is the cause of the disease. Unfortunately, we were not able to clarify whether one of the both mutations occurred "de novo", as no DNA samples from putative affected relatives of the patient were available.

Although we did not find compound heterozygous mutations comprising point mutations and genomic rearrangements in HNPCC patients, those biallelic inactivations may not be infrequent: assuming that the minimal allele frequency of genomic rearrangements in pathogenic MMR gene mutations is 15% [14,16,17,20,22,38,39], the probability of random compound heterozygous mutations to include at least one genomic rearrangement is 27.75%. Of 31 individuals reported as homozygote or compound heterozygote for *MLH1*, *MSH2*, *MSH6* or *PMS2* mutations thus far [34,35], none carries a genomic rearrangement. This may be due to screening for genomic rearrangements not being a standard procedure until recently. Furthermore, biallelic mutations have been described in very young patients, which may be the reason for our failure to identify individuals with two pathogenic mutations in our 137 HNPCC patients. Only one of our patients was younger than 20 years. Therefore, it seems reasonable to search for additional genomic rearrangements in very young patients carrying a pathogenic point mutation or an unclassified variant. Because of the significant clinical consequence implied in the identification of all causative germline mutations and for predictive molecular testing in the families, the data suggest testing for genomic rearrangement as a standard

Table 1
Identified genomic rearrangements in MMR genes

Patient ID	Patient group[a]	Criteria of the Bethesda guidelines	Point mutation, MMR gene	Affected gene	Rearrangement
120	1	4	–	MLH1	EX17del
5704	1	1	–	MLH1	EX7_8del
5781	1	2, 4	–	MLH1	EX14_15del
4521	1	2	–	MSH2	EX1_3del
4778	1	1	–	MSH2	EX3_6del
5137	1	4	–	MSH2	EX8_16del
5277	1	2	–	MSH2	EX1_16del
5604	1	2–4	–	MSH2	EX1_3del
5948	1	4	–	MSH2	EX7del
6809	1	2	–	MSH2	EX1_16del
7955	1	2–4	–	MSH2	EX1_8del
8646	1	3, 4	–	MSH2	EX9_10del
9047	1	4	–	MSH2	EX1_6del
2430	3	3, 4	c.1853A > G, (p.K618R), MLH1	MLH1	EX1_15del

MMR, mismatch repair.
[a] Patient groups as defined in Section 2.

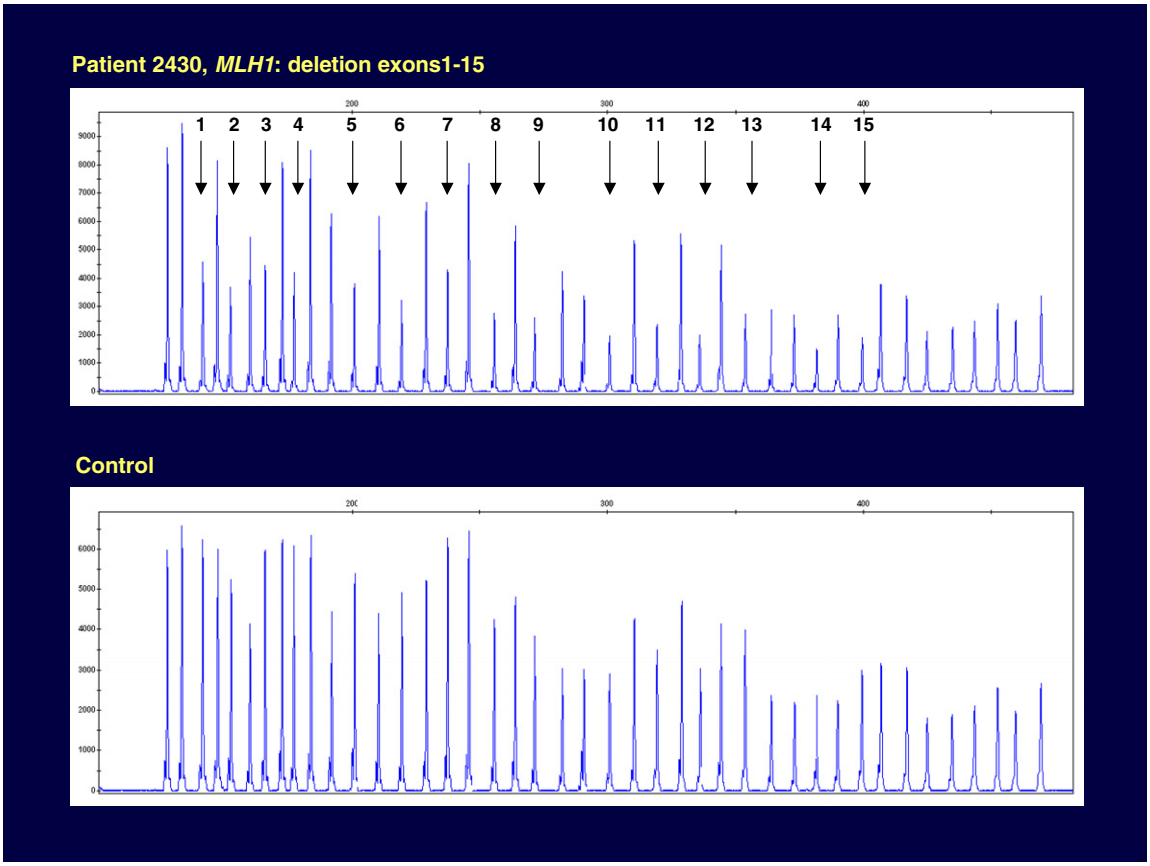

Fig. 1. Result of MLPA analysis (EX1_15del of *MLH1*) in patient 2430.

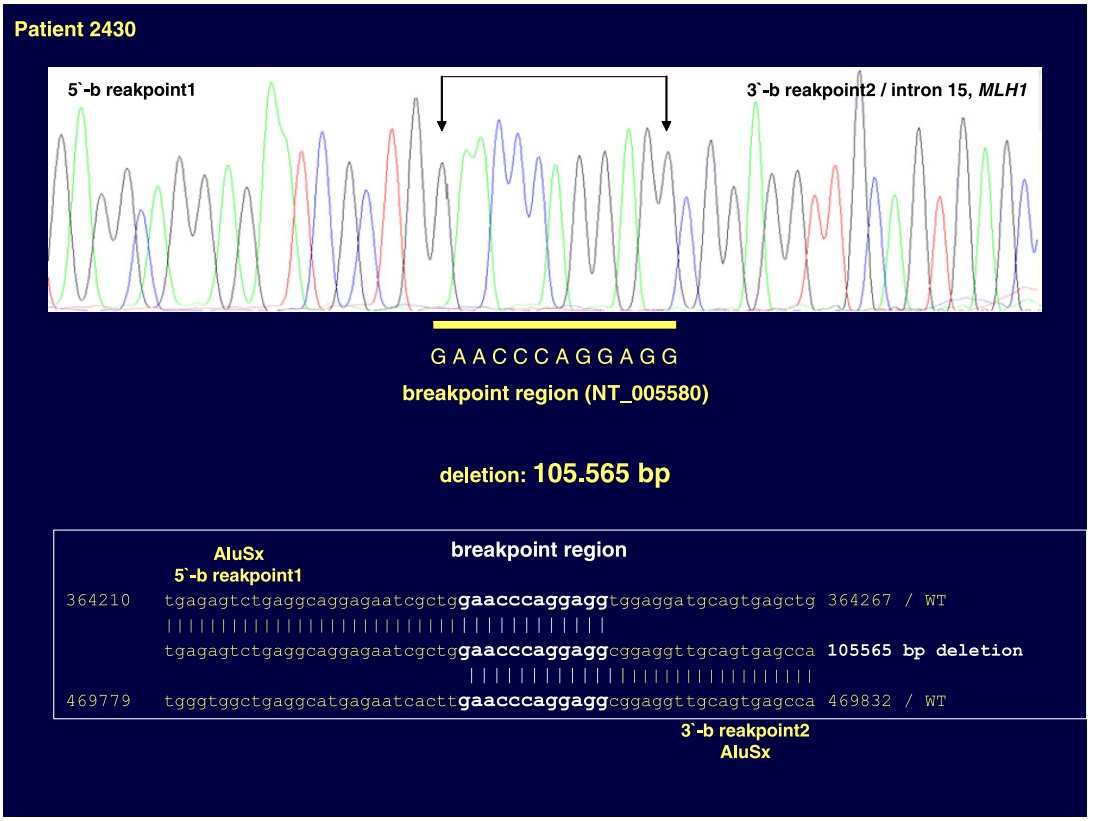

Fig. 2. Result of breakpoint analysis (EX1_15del in *MLH1*) in patient 2430. EX1_15del in *MLH1* in patient 2430 was verified by breakpoint analysis and long-range PCR, which identified a 105.565 bp deletion between two Alu repeats of the Sx type family.

procedure in MMR gene diagnostics of all index patients suspected for HNPCC.

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