A novel *MSH2* germline mutation in homozygous state in two brothers with colorectal cancers diagnosed at the age of 11 and 12 years

Annegret Müller[1§], Hans K. Schackert[2§], Bettina Lange[1], Josef Rüschoff[3], Laslow Füzesi[4], Joerg Willert[5], Peter Burfeind[6], Parantu Shah[7], Heinz Becker[1], Joerg Thomas Epplen[8], Susanne Stemmler[8]

[1]Department of General Surgery, University of Göttingen, Germany;
[2]Department of Surgical Research, Technische Universität Dresden, Germany;
[3]Institute of Pathology, Klinikum Kassel, Germany;
[4]Department of Pathology, University of Göttingen, Germany;
[5]Department of Medicine, University of Bochum, Germany;
[6] Institute of Human Genetics, University of Göttingen, Germany;
[7]Structural and Computational Biology Unit, EMBL Heidelberg, Germany;
[8]Department of Human Genetics, University of Bochum, Germany

§ contributed equally to this paper

*Correspondence to
PD Dr. A. Müller
Department of General Surgery
University of Göttingen
Robert-Koch-Str. 40
37075 Göttingen, Germany
Tel: +49-551-39-6104
Fax: +49-551-396106
e-mail dramueller@aol.com

Author: Müller et al.
Running head: Homozygous *MSH2* mutation &childhood cancer

Hereditary nonpolyposis colorectal cancer syndrome (HNPCC) is caused by heterozygous germline mutations in DNA mismatch repair genes (MMR), (*MSH2*, *MLH1*, *MSH6* and *PMS2)* and it is inherited in an autosomal dominant pattern with high penetrance. Several patients have been reported carrying bi-allelic MMR gene mutations and whose phenotype resembled a syndrome with childhood malignancies including hematological malignancies, brain and colorectal tumors. This phenotype is similar to the tumor spectrum of MMR knockout mice. Herein we describe two brothers of healthy consanguineous parents from Pakistan, who had developed two and three colorectal cancers at the ages of 11 and 12 years, respectively, and less than 30 polyps. Tumor specimens were microsatellite instable (MSI-H), and expression of MSH2 and MSH6 was lost. Mutation analyses of DNA samples from both patients revealed a novel homozygous c.2006-5T>A mutation in intron 12 of the *MSH2* gene. This phenotype of the brothers is unusual as they did not develop hematological malignancies nor brain tumors at an older age of presentation than other patients with homozygous *MSH2* mutations. The milder phenotype may be due to the expression of low amounts of MSH2 protein with reduced activity.

KEY WORDS: colorectal cancer, childhood, homozygous mutation, MMR system

INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC) is the most common form of hereditary colorectal cancer (for review see [Lynch and de la Chapelle, 2003]). Germline mutations in one of the DNA mismatch repair (MMR) genes, *MSH2* or, and less frequently in the genes *MSH6*, *PMS2* or *MLH3* cause the syndrome in the vast majority of cases [Aaltonen et al., 1998; Evans et al., 1997; Salovaara et al., 2000; Wu et al., 2001]. Patients who are heterozygous for a MMR gene mutation are prone to develop colorectal cancers, and a broad spectrum of malignancies outside the intestinal tract at an average age of ~45 years [Lynch and de la Chapelle, 2003].

Colorectal cancer in childhood is very rare. Recently, HNPCC families have been reported with family members sharing homozygous or compound heterozygous mutations in MMR genes [Bougeard et al., 2003; De Rosa et al., 2000; Hedge et al., 2005; Gallinger et al., 2004; Menko et al., 2004; Ricciardone et al., 1999; Trimbath et al., 2001; Vilkki et al., 2001; Wang et al., 1999; Whiteside et al., 2002]. The tumor spectrum in these families comprises hematological malignancies, brain tumors and gastrointestinal tumors, which resembles the phenotype of *MSH2* knockout mouse models [Edelmann and Edelmann, 2004].

Herein we report the unusual phenotype of two brothers aged 11 and 12 years old, diagnosed with multiple colorectal cancers and polyps suggestive of a familial adenomatous polyposis coli (FAP), who carry a novel homozygous mutation in the *MSH2* gene (c.2006-5T>A). Further examinations of these children were negative for

hematological malignancies or brain tumors, suggesting that a homozygous *MSH2* mutation may cause a milder phenotype than previously recognized.

MATERIAL AND METHODS

Patient data

The 11-year-old patient was admitted to the clinic due to abdominal pain and hematochezia. Further examinations revealed multiple polyps in the duodenum, colon and rectum (high-grade and carcinoma *in-situ* tubulous adenoma) as well as one cancer at the left flexure of the colon. Additionally, the patient showed multiple café-au-lait spots.

Physical examination, upper endoscopy and colonoscopy of the parents and the 6-year-old brother were negative, whereas in the 12-year-old brother we found three carcinomas (ascending colon, descending colon and rectum) as well as multiple polyps in the colon (less than ten), two polyps in the duodenum and multiple café-au-lait spots. The surgical therapy consisted of a colectomy and a mucosal proctectomy with ileoanal anastomosis (J-pouch) in both brothers. The massive polyp at the papilla of Vater and the duodenal tumors were removed endoscopically.

Family history documented that the parents from Pakistan were consanguineous. An 11-month-old brother was too young to undergo colonoscopy, but ultrasound examination of the abdomen was negative for tumors. The family was given genetic counseling and written informed consent was obtained for molecular diagnostics. The study was approved by the local ethics committee of the University of Dresden.

Microsatellite analysis and Immunohistochemistry

Microsatellite instability in tumor tissue was analyzed by applying the reference marker panel for the evaluation of microsatellite instability (MSI) in colorectal cancer (BAT25, BAT26, D2S123, D5S346, and D17S250) of the National Cancer Institute/International Collaborative Group on HNPCC (NCI/ICG-HNPCC) [Boland et al., 1998].

Immunohistochemical staining was performed for MLH1, PMS2, MSH2 and MSH6 on 5-µm-thick, formalin-fixed, paraffin-embedded sections from tumors of the patients as described previously [Plaschke et al., 2002].

Mutation analysis

Mutation analysis of the coding exons of *MSH2* (16 exons) and flanking introns was conducted by polymerase chain reaction (PCR) amplifications and DNA sequencing. DNA was extracted from EDTA-anticoagulated peripheral blood by using a standard salting-out method [Miller et al., 1988]. PCR reactions were performed in a total volume of 12.5 µl, containing 50 ng DNA, 200 mmol of each dNTP, 1 to 2 mmol $MgCl_2$, 10 pmol of each primer and 0.4 U Taq polymerase (Genecraft, Münster, Germany). PCR was performed in a Biometra thermal cycler (Whatman, Göttingen, Germany). After two initial cycles at 6 °C and 3 °C above the annealing temperature (56 °C), 28 cycles of 95 °C (30 sec), annealing temperature (30 sec) and 72 °C (30 sec) were run.

DNA samples of each exon of the *MSH2* gene were sequenced using the BigDye cycle sequencing Kit (Applied Biosystems, Darmstadt, Germany) as described in the manufacturer's instructions on an automated ABI PRISM 377 DNA Sequencer (Applied Biosystems, Darmstadt, Germany). Mutation nomenclature is according to [den Dunnen and Antonarakis, 2001].

To investigate alternative splicing, mRNA was isolated from peripheral blood leukocytes and cDNA was synthesized using a RT-PCR assay as described elsewhere [Jagiello et al., 2003]. Primers amplifying exon 13 of the cDNA and the adjacent sequence of exons 12 and 14 were applied and PCR reaction was performed in a total volume of 25 µl under the same conditions as described above. 5 µl of each PCR product were separated on 2.5% agarose gels in 1x TBE buffer (30 min, 200 V) and visualized using ethidium bromide.

The gel image was loaded using Genetools software (Synoptics, Cambridge, England) and the quantity of DNA was analyzed after manual adjustment. In addition, cDNA bands were cut out of the gel and were sequenced as described above.

To analyze the structural impact of the mutation we used Modeller [Martin-Renom et al., 2000] to generate various models of MSH2 with a valine preceding the conserved “P-loop” nucleotide binding motif. Models were chosen based on Modellers objective function and their quality was checked using ANOLEA force field [Melo and Feytmans, 1997].

RESULTS

Microsatellite analysis of one tumor specimen of each patient revealed a MSI-H status, where 5/5 markers showed additional bands compared to the corresponding normal tissue specimens (data not shown). Immunohistochemistry did not detect expression of MSH2 and MSH6 in the tumor and surrounding normal tissue (data not shown). FAP was excluded by *APC* gene analysis (data not shown).

Mutation analyses showed a c.2006-5T>A transversion in intron 12 of the *MSH2* gene in both alleles of both patients. In addition, the brothers carried the homozygous

C allele of the c.2006-6T>C polymorphism. The parents and their 6-year and 11-month-old children carried the disease-causing allele in a heterozygous state. Since this mutation has not been described previously, we investigated whether it results in alternative splicing of the *MSH2* transcript. RT-PCR and agarose gel electrophoresis of the cDNAs obtained from both affected brothers revealed the existence of two *MSH2* transcripts of different lengths. Sequence analysis showed skipping of exon 13 in the shorter fragment, which is predicted to result in a frameshift, and creation of a premature stop codon at position 676, thus producing a truncated protein. The c.2006-5T>A substitution in intron 12 apparently creates an aberrant splice site (AG). This splice site mutation causes the insertion of the last three intronic bases TAG at position 2006 of the cDNA into the transcript and is predicted to result in the insertion of the additional amino acid valine into the protein. Semi-quantitative analysis of the cDNA of both brothers revealed that roughly two thirds of the transcript had skipped exon 13, while the transcript that included exon 13 comprised about one third of the *MSH2* transcripts and included the TAG insert, which is predicted to result in the insertion of valine into the protein. We detected no wild type cDNA sequences in the affected brothers.

DISCUSSION

Colorectal cancer at young age is rare, and in addition to family history, is a sign that the malignancy may be familial [Umar et al., 2004]. The median age of diagnosis of colorectal cancers in patients with FAP is 35 years, and ten years later in patients with HNPCC [Lynch and de al Chapelle, 2003]. The mode of inheritance of both FAP and HNPCC is autosomal dominant with high penetrance. Only a few reports on young

patients with MMR gene mutations exist [Bougeard et al., 2003; De Rosa et al., 2000; Gallinger et al., 2004; Menko et al., 2004; Ricciardone et al., 1999; Trimbath et al., 2001; Vilkki et al., 2001; Wang et al., 1999; Whiteside et al., 2002]. Notably, these patients are homozygotes or compound heterozygotes for mutations in the *MSH2, MLH1, MSH6* or *PMS2* genes. In contrast to patients who are heterozygous for MMR gene mutation, their phenotype is different since they develop primarily hematological malignancies and brain tumors, and less frequently gastrointestinal tumors. This is in part similar to the Muir-Torre and Turcot syndromes [Cohen et al., 1992; Hamilton et al., 1995] and resembles the phenotype of MMR knockout mice [Edelmann and Edelmann, 2004]. Interestingly, there are great differences in the age of disease onset in patients carrying bi-allelic mutations of different MMR genes. For example, the median age at diagnosis of tumors is 5 years (range 1-22 years) in bi-allelic *MLH1* mutation carriers, 8 years (range 5-12 years) in *MSH6* mutation carriers and 13.5 years (range 4-24 years) in *PMS2* mutation carriers.

Bi-allelic mutations in *MSH2* have been described in three patients of two families [Bougeard et al., 2003; Whiteside et al., 2002]. In contrast to the patients described here, these were remarkably young. One patient presented with T-lymphocyte acute lymphatic leukemia at the age of 24 months and in a second family two siblings died of a T cell mediastinal lymphoma and temporal glioblastoma at ages 15 months and 4 years, respectively. No gastrointestinal tumors were reported. Both children from the second family carried an EX1_6del and a c.454delA of *MSH2,* which were predicted to result in truncated proteins. The 11-month-old patient from family 1 carried a homozygous IVS10-1G>A splice site mutation of *MSH2*, which results in skipping of

exon 11 and exon 12 and a truncated protein. The mutation reported here results in two transcripts. The more abundant cDNA species lacks exon 13 and is predicted to code for a truncated protein. Notably, low amounts of aberrant exon 13 skipping have been described in persons carrying homozygous wild type alleles of *MSH2* [Mori et al., 1997; Xia et al., 1996]. The less abundant cDNA species had the c.2005_2006insTAG, which predicts p.T668_G669insV.This sequence is part of the highly conserved nucleotide-binding motif GPNMGGKS (residues 669-676) in MSH2 which forms a P-loop and marks the ATP-binding site [Obmolova et al., 2000].

Remarkably, the phenotype of the two brothers differs from reported phenotypes as the patients reported here did not develop hematological malignancies and brain tumors and were much older than the three previously described patients. Although not detectable by immunohistochemistry, the expression of low amounts of variant MSH2 protein containing the p.T668_G669insV mutation should to be assumed, based on the cDNA species that we detected. We hypothesize that there is a residual activity of this variant MSH2 protein, which results in a milder phenotype. To analyze the functional impact of the mutation, we used homology modeling based on the similar three-dimensional structures of MutS proteins from *Thermus aquaticus* and *E. coli* by requiring the essential solvent accessibility of the P-loop. It indeed suggests an outcome that does not appear to directly affect the active site. The inserted valine takes the position of the last residue in the beta-strand preceding the conserved P-loop (threonine in the wild type). As a consequence, the entire beta-strand is shifted by one hydrogen bond. This effectively inserts an amino acid on the other side of the protein (away from the active site) and does not interfere with nucleotide binding. This may

explain the distinct phenotype in the patients when compared to other patients with mutations in this gene.

ACKNOWLEDGEMENT

We thank Peer Bork from the Structural and Computational Biology Unit, EMBL Heidelberg, Germany, for his expertise in computational modeling of protein structures. We thank Christian Pilarsky from the Department of Surgery, Dresden University of Technology, for performing the semi-quantitative analysis of the cDNA. Contract grant sponsor: Deutsche Krebshilfe (DKH, German Cancer Aid); Contract grant number: "Familiärer Darmkrebs" 70-3032

REFERENCES

Aaltonen LA, Salovaara R, Kristo P, Canzian F, Hemminki A, Peltomaki P, Chadwick RB, Kaariainen H, Eskelinen M, Jarvinen H, Mecklin JP, de la Chapelle A. 1998. Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease. N Engl J Med 338:1481-1487.

Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodriguez-Bigas MA, Fodde R, Ranzani GN, Srivastava S. 1998. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 58:5248-5257.

Bougeard G, Charbonnier F, Moerman A, Martin C, Ruchoux MM, Drouot N, Frebourg T. 2003 Early onset brain tumor and lymphoma in MSH2-deficient children. Am J Hum Genet 72:213-216.

Cohen PR, Kohn SR, Kurzrock R. 1991. Association of sebaceous gland tumors and internal malignancy: the Muir-Torre syndrome. Am J Med 90:606-613.

den Dunnen JT, Antonarakis E. 2001. Nomenclature for the description of human sequence variations. Hum Genet 109:121-124.

De Rosa M, Fasano C, Panariello L, Scarano MI, Belli G, Iannelli A, Ciciliano F, Izzo P. 2000. Evidence for a recessive inheritance of Turcot's syndrome caused by compound heterozygous mutations within the PMS2 gene. Oncogene 19:1719-1723.

Edelmann L and Edelmann W. 2004. Loss of DNA mismatch repair function and cancer predisposition in the mouse: animal models for human hereditary nonpolyposis colorectal cancer. Am J Med Genet C Semin Med Genet 129:91-99.

Evans DG, Walsh S, Jeacock J, Robinson C, Hadfield L, Davies DR, Kingston R. 1997. Incidence of hereditary non-polyposis colorectal cancer in a population-based study of 1137 consecutive cases of colorectal cancer. Br J Surg 84:1281-1285.

Gallinger S, Aronson M, Shayan K, Ratcliffe EM, Gerstle JT, Parkin PC, Rothenmund H, Croitoru M, Baumann E, Durie PR, Weksberg R, Pollett A, Riddell RH, Ngan BY, Cutz E, Lagarde AE, Chan HS. 2004. Gastrointestinal cancers and neurofibromatosis type 1 features in children with a germline homozygous MLH1 mutation. Gastroenterology 126:576-585.

Hamilton SR, Liu B, Parsons RE, Papadopoulos N, Jen J, Powell SM, Krush AJ, Berk T, Cohen Z, Tetu B. 1995. The molecular basis of Turcot's syndrome. N Engl J Med 332:839-847.

Hedge MR, Chong B, Blazo ME, Chin LH, Ward PA, Chintagumpala MM, KIm JY, Plon SE, Richards CS. 2005. A homozygeous mutation in MSH6 causes Turcots syndrome. Clin Cancer Res 11:4689-4693.

Jagiello P, Hammans C, Wieczorek S, Arning L, Stefanski A, Strehl H, Epplen JT, Gencik M. 2003. A novel splice site mutation in the TRIM37 gene causes mulibrey nanism in a Turkish family with phenotypic heterogeneity. Hum Mutat 21:630-635.

Lynch HT and de la Chapelle A. 2003. Hereditary colorectal cancer. N Engl J Med 348:919-932.

Menko FH, Kaspers GL, Meijer GA, Claes K, van Hagen JM, Gille JJ. 2004. A homozygous MSH6 mutation in a child with cafe-au-lait spots, oligodendroglioma and rectal cancer. Fam Cancer 3:123-127.

Marti-Renom MA, Stuart AC, Fiser A, Sanchez R, Melo F, Sali A. 2000. Comparative protein structure modeling of genes and genomes. Annu Rev Biophys Biomol Struct 29: 291-325.

Melo F and Feytmans E. 1997. Novel knowledge-based mean force potential at atomic level. J Mol Biol 267: 207-222.

Miller SA, Dykes DD, Polesky HF. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res.16:1215.

Mori Y, Shiwaku H, Fukushige S, Wakatsuki S, Sato M, Nukiwa T, Horii A. 1997. Alternative splicing of hMSH2 in normal human tissues. Hum Genet 99:590-595.

Obmolova G, Ban C, Hsieh P, Yang W. 2000. Crystal structures of mismatch repair protein MutS and its complex with a substrate DNA. Nature 12:703-710.

Plaschke J, Krüger S, Pistorius S, Theissig F, Saeger HD, Schackert HK. 2002. Involvement of hMSH6 in the development of hereditary and sporadic colorectal cancer revealed by immunostaining is based on germline mutations, but rarely on somatic inactivation. Int J Cancer 97:643-648.

Ricciardone MD, Ozcelik T, Cevher B, Ozdag H, Tuncer M, Gurgey A, Uzunalimoglu O, Cetinkaya H, Tanyeli A, Erken E, Ozturk M. 1999. Human MLH1 deficiency predisposes to hematological malignancy and neurofibromatosis type 1. Cancer Res 59:290-293.

Salovaara R, Loukola A, Kristo P, Kaariainen H, Ahtola H, Eskelinen M, Harkonen N, Julkunen R, Kangas E, Ojala S, Tulikoura J, Valkamo E, Jarvinen H, Mecklin JP, Aaltonen LA, de la Chapelle, A. 2000. Population-based molecular detection of hereditary nonpolyposis colorectal cancer. J Clin Oncol 18:2193-200.

Trimbath JD, Petersen GM, Erdman SH, Ferre M, Luce MC, Giardiello FM. 2001. Cafe-au-lait spots and early onset colorectal neoplasia: a variant of HNPCC? Fam Cancer 1:101-105.

Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle, Ruschoff J, Fishel R, Lindor NM, Burgart LJ, Hamelin R, Hamilton SR, Hiatt RA, Jass J, Lindblom A, Lynch HT, Peltomaki P, Ramsey SD, Rodriguez-Bigas MA, Vasen HF, Hawk ET, Barrett JC, Freedman AN, Srivastava S. 2004. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst 96:261-268.

Vilkki S, Tsao JL, Loukola A, Poyhonen M, Vierimaa O, Herva R, Aaltonen LA, Shibata D. 2001. Extensive somatic microsatellite mutations in normal human tissue. Cancer Res 61:4541-4544.

Wang Q, Lasset C, Desseigne F, Frappaz D, Bergeron C, Navarro C, Ruano E, Puisieux A. 1999. Neurofibromatosis and early onset of cancers in hMLH1-deficient children. Cancer Res 59:294-297.

Whiteside D, McLeod R, Graham G, Steckley JL, Booth K, Somerville MJ, Andrew SE. 2002. A homozygous germ-line mutation in the human MSH2 gene predisposes to hematological malignancy and multiple cafe-au-lait spots. Cancer Res 62:359-362.

Wu Y, Berends MJ, Sijmons RH, Mensink RG, Verlind E, Kooi KA, van der Sluis T, Kempinga C, van der Zee AG, Hollema H, Buys CH, Kleibeuker JH, Hofstra RM. 2001. A role for MLH3 in hereditary nonpolyposis colorectal cancer. Nat Genet 29:137-138.

Xia L, Shen W, Ritacca F, Mitri A, Madlensky L, Berk T, Cohen Z, Gallinger S, Bapat B. 1996. A truncated hMSH2 transcript occurs as a common variant in the population: implications for genetic diagnosis. Cancer Res 56:2289-2292.