Genome-wide copy neutral LOH is infrequent in familial and sporadic microsatellite unstable carcinomas

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Abstract Mismatch repair deficiency in tumors can result from germ line mutations in one of the mismatch repair (MMR) genes (*MLH1, MSH2, MSH6* and *PMS2*), or from sporadic promoter hypermethylation of *MLH1*. The role of unclassified variants (UVs) in MMR genes is subject to debate. To establish the extend of chromosomal instability and copy neutral loss of heterozygosity (cnLOH), we analyzed 41 archival microsatellite unstable carcinomas, mainly colon cancer, from 23 patients with pathogenic MMR mutations, from eight patients with UVs in one of the MMR genes and 10 cases with *MLH1* promoter hypermethylation. We assessed genome wide copy number abnormalities and cnLOH using SNP arrays. SNP arrays overcome the problems of detecting LOH due to instability of polymorphic microsatellite markers. All carcinomas showed relatively few chromosomal aberrations. Also cnLOH was infrequent and in Lynch syndrome carcinomas usually confined to the locus harbouring pathogenic mutations in *MLH1, MSH2* or *PMS2* In the carcinomas from the MMR-UV carriers such cnLOH was less common and in the carcinomas with *MLH1* promoter hypermethylation no cnLOH at *MLH1* occurred. MSI-H carcinomas of most MMR-UV carriers present on average with more aberrations compared to the carcinomas from pathogenic MMR mutation carriers, suggesting that another possible pathogenic MMR mutation had not been missed. The approach we describe here shows to be an excellent way to study genome-wide cnLOH in archival mismatch repair deficient tumors.

Keywords Lynch syndrome · HNPCC · MSI-H · Chromosomal instability · Copy neutral loss of heterozygosity · Mismatch repair (MMR) genes · Unclassified variants · *MLH1* hypermethylation · SNP array

Abbreviations

CGH	Comparative genomic hybridization
CIN	Chromosomal instability
CNA	Copy number aberrations
cnLOH	Copy neutral loss of heterozygosity
CRC	Colorectal cancer
FFPE	Formalin-fixed paraffin-embedded
GCS	Gene call score
GTS	Gene train score
IHC	Immunohistochemistry
LOH	Loss of heterozygosity
LP	Linkage panels
MMR	Mismatch repair
MSI	Microsatellite instability
MSI-H	Microsatellite instability
MSS	Microsatellite stable
rGCS	Relative gene call score
SRO	Smallest region of overlap
UVs	Unclassified variants

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Introduction

In colorectal cancer (CRC) there are two classical pathways that direct tumorigenesis: microsatellite instability (MSI or MIN) and chromosomal instability (CIN). MSI results from a defective DNA mismatch repair (MMR) system and therefore characterises tumors from patients with Lynch syndrome (previously HNPCC, hereditary nonpolyposis colorectal cancer). In addition 15% of sporadic CRC displays MSI due to *MLH1* promoter hypermethylation [1–3]. Tumor cells with abrogated MMR function accumulate small deletions and insertions in stretches of short repetitive DNA sequences distributed throughout the genome. These mutations lead to frameshifts within coding sequences and thus inactivation of genes, thereby contributing to tumor development and progression [4–6]. MSI carcinomas most often show a diploid or near-diploid genome [7], while up to 73% of sporadic CRC tumors show aneuploidy, the equivalent of a gross amount of CIN [8]. In sporadic microsatellite unstable (MSI-H) carcinomas the most frequent aberrations are gains of chromosome 8, 12 and 13 while chromosomal losses occurred predominantly at 15q14 [9]. In sporadic microsatellite stable (MSS) CRC, CIN is characterized by losses and amplifications of arms of, or complete, chromosomes [10–12]. In general, physical loss of chromosomes 17p and 18q, and gain at 8q, 13q, and 20 occur at early stages during the transition from adenoma to carcinoma, whereas loss of 4p is associated with transition from Dukes' A to B–D. Chromosomal loss of 8p and gain of 7p and 17q is reported to be associated with the transition from primary carcinoma to local and distal metastases. Loss of 14q and gains of 1q, 11, 12p, and 19 are considered late events [13, 14]. Both chromosomes 5 and 17p are more often targeted by copy number neutral LOH than by copy number variations [15, 16].

Clinically, the uncertainty about the contribution of an MMR unclassified variant (MMR-UV) to the risk of developing cancer is a major problem. While carriers of a pathogenic MMR mutation are at increased risk, those with an MMR-UV could also represent rare variants without increased risk of cancer. For pathogenic MMR carriers, clinical geneticists offer pre-symptomatic testing for the detection of neoplasia at an early stage. For patients carrying an MMR-UV with unproven pathogenicity, offering pre-symptomatic testing is difficult.

Since 2001 evidence for differences between sporadic and familial MSI-H carcinomas with respect to both genotype and phenotype is accumulating. [17, 18] To expand this knowledge we determined the possible difference in genomic tumor profiles of patients with pathogenic MMR mutations, MMR-unclassified variants and of sporadic carcinomas with *MLH1* promoter hypermethylation.

Material and methods

Thirty-seven formalin-fixed paraffin-embedded (FFPE) microsatellite unstable (MSI-H) colorectal tumors from 37 patients selected from the pathology archives were included in our study. Corresponding histological normal tissue from 30 of these patients and leukocyte DNA for seven patients was available. In addition, four FFPE endometrial carcinomas with corresponding normal DNA were analyzed. Thirty-one of these samples originated from patients with familial MMR deficiency; the following mutation carriers were included: 11 *MLH1* (6 pathogenic, 5 UVs), 10 *MSH2* (7 pathogenic, 3 UVs), 5 *MSH6* (all pathogenic), and 5 *PMS2* (all pathogenic) mutation carriers. One *MLH1*–UV carrier also showed a mono-allelic G382D mutation in *MUTYH* and one *PMS2* carrier showed a V878A UV in *MSH6* as well. A subset of these cases has been reported previously [19, 20]. The mean age at diagnosis of cancer was 49 years for the pathogenic MMR mutation carriers, and 43 years for the MMR-UV carriers. Clinical and mutation data are given in Table 1. The additional 10 samples originate from 10 patients that present with sporadic MMR deficient right sided (RST) colon carcinomas based on *MLH1* promoter hypermethylation with a mean age of 76 years. The study was approved by the Medical Ethical Committee of the LUMC (protocol P01-019) and the tumors were analyzed following the guidelines described in the code for proper secondary use of human tissue established by the Dutch federation of medical sciences (http://www.federa.org/).

MSI analysis and immunohistochemistry (IHC) of the MMR genes

MSI analysis and immunohistochemical staining of the MMR proteins was performed as described by de Jong et al. [19].

DNA isolation

Normal and tumor tissue was selected by a pathologist (HM), guided with microscopy of a hematoxylin eosin-stained slide. DNA of the selected tissue was extracted from FFPE material as described [19]. The DNA was subsequently cleaned up using protein precipitation solution (Promega, Leiden, The Netherlands) and 2-propanol precipitation. Leukocyte DNA was obtained by salting out precipitation. DNA concentrations were measured using picogreen (Invitrogen-Molecular Probes, Carlsbad, CA, USA).

Hypermethylation analysis of the *MLH1* promoter

The *MLH1* promoter hypermethylation status of the five *MLH1*-UVs and the 10 sporadic MSI-H right-sided tumors

Table 1 Characteristics of pathogenic MMR mutation, MMR-UV carriers and patients with sporadic MSI-H carcinomas

Sample ID	Tumor location	Stage[a]	Age of onset	MSI	IHC-MLH1	IHC-MSH2	IHC-MSH6	IHC-PMS2	Mutated gene	Exon
1	Colon right	3	47	H	0	+	0	0	*MLH1*[b]	3
3	Colon right	3	30	H	0	+	+	0	*MLH1*	12
4	Colon right	2	47	H	0	+	0	0	*MLH1*	16
5	Colon right	2	68	H	0	+	+	0	*MLH1*	16
6	Colon right	3	45	H	0	+	0	na	*MLH1*	10
7	Colon right	3	38	H	0	+	+	0	*MLH1*	1
8	Colon uns	na	54	H	+	0	0	na	*MSH2*	7
9	Colon left	2	67	H	+	0	0	na	*MSH2*	9
10	Colon right	3	58	H	+	0	0	+	*MSH2*	3
11	Colon right	2	38	H	+	0	0	+	*MSH2*	3
12	SmB	2	39	H	+	0	0	+	*MSH2*	13
13	Colon left	1	26	H	+	0	0	+	*MSH2*	7
14	Colon uns	2	49	H	+	0	0	+	*MSH2*	12
15	Colon uns	1	47	H	+	+	0	+	*MSH6*	9
16	Colon left	1	44	H	+	+	0	na	*MSH6*[c]	3
17	Colon right	3	54	H	+	+	0	na	*MSH6*[d]	4
18	Colon right	2	59	H	+	0	0	+	*MSH6*	4
19	Colon left	3	49	H	+	+	0	+	*MSH6*	4
20	Colon right	2	42	H	+	+	0	0	*PMS2*	11
21	Colon right	2	46	H	+	+	+	0	*PMS2*	–
22	Colon left	1	52	H	0	+	0	0	*PMS2*	3_7
23	EC	Na	81	H	+	+	0	na	*PMS2*	10
24	EC	Na	47	H	+	+	0	0	*PMS2*[e]	8
25a	Colon right	2	54	H	0	+	0	na	*MLH1* UV[f]	3
27	Colon right	2	38	H	0	+	+	na	*MLH1* UV	16
28a	Colon left	2	36	H	+	+	+	+	*MLH1* UV	1
29	EC	Na	34	H	+	+	+	+	*MLH1* UV	1
31	EC	Na	45	H	+	+	+	+	*MLH1* UV	1
32	Colon left	2	48	H	+	+	0	+	*MSH2* UV	15
33	Colon left	3	36	H	+	+	+	na	*MSH2* UV	11
34	Colon left	3	53	H	+	+	+	+	*MSH2* UV	14
S10	Colon right	2	80	H	0	+	+	0	–	
S16	Colon right	3	80	H	0	+	+	0	–	
S19	Colon right	2	75	H	0	+	+	0	–	

Table 1 continued

Sample ID	Tumor location	Stage[a]	Age of onset	MSI	IHC-MLH1	IHC-MSH2	IHC-MSH6	IHC-PMS2	Mutated gene	Exon
S20	Colon right	3	75	H	0	+	+	0	–	
S32	Colon right	3	76	H	0	+	+	0	–	
S39	Colon right	2	90	H	0	+	+	0	–	
S43	Colon right	2	61	H	0	+	+	0	–	
S51	Colon right	2	69	H	0	+	+	0	–	
S69	Colon right	2	76	H	0	+	+	0	–	
S78	Colon right	3	82	H	na	+	+	0	–	

Sample ID	DNA nucleotide change	Predicted protein change	Predicted biological type	Clinical status	Missing LP (1,2,3,4)	CNA Gain/*loss*	cnLOH	Segment size[g] CNA	cnLOH	Total
1	c.298 C > T	p.Arg100X	Nonsense	Path	2			0	0	0
3	c.1046dupT	p.Pro350fsX12	Frameshift	Path	2		3p26.3–14.1 19p13.3	0	22	22
4	c.1852_1854del 3	p.Lys618del	1 amino acid deletion	Path	–		3p25.1–21.1; 3q27.1	0	14	14
5	c.1852_1854del 3	p.Lys618del	1 amino acid deletion	Path	4		3p26.3–21.31	0	16	16
6	c.806C > G	p.Ser269X	Nonsense	Path	–		3p26.3–21.31	0	16	16
7	c.18_34del17	p.Val7fsX18	Frameshift	Path	4	**3q26.6** **9p24.3–22.3**	3p26.3–21.31	6	16	22
8	c.379del T	p.?	Frameshift	Path	–		2p25.3–13.3 6q24.3–25.3	0	23	23
9	c.1408_1410delGTAinsCT	p.Val471fsX11	Frameshift	Path	3			0	0	0
10	c.367-?_645+?del	p.?	Exon(s) deletion	Path	–	*2p21–16.2*	2p22.1	3	1	4
11	c.367-?_645+?del	p.?	Exon(s) deletion	Path	3		2p25.3–14 6p25.3–22.3	0	26	26
12	c.2006-?_2210+?del	p.?	Exon(s) deletion	Path	–		17q23.2–25.3	0	8	8
13	c.1221_1222delCT	p.Tyr408fsX8	Frameshift	Path	1,2			0	0	0
14	c.1861C > T	p.Arg621X	Nonsense	Path	–	*20p13–20q13.32*	6q24.2–25.2	19	4	23
15	c.4001G > A	p.Arg1334Gln	Splice site	Path	1			0	0	0
16	c.467C > G	p.Ser156X	Nonsense	Path	–		22q arm	0	12	12
17	c.1444C > T	p.Arg482X	Nonsense	Path	–	*9p24.3–24.1*	12p arm 19q13.11–13.43	3	22	25
18	c.1784delT	p.Leu595fsX15	Frameshift	Path	–			0	0	0
19	c.2191C > T	p.Gln731X	Nonsense	Path	–		15q arm	0	28	28
20	c.1882C > T	p.Arg628X	Nonsense	Path	1	**19q13.31–13.41**	15q21.3–22.2	4	7	11

Table 1 continued

Sample ID	DNA nucleotide change	Predicted protein change	Predicted biological type	Clinical status	Missing LP (1,2,3,4)	CNA Gain/loss	cnLOH	Segment size[g] CNA	cnLOH	Total
21	2 kb insertion intron 7	p.?	Intron variant	Path	–		7p arm	0	18	18
22	deletion exon 3_7	p.?	Exon(s) deletion	Path	–		2p25.3–15	0	17	17
23	deletion exon 10	p.?	Exon(s) deletion	Path	–		7p22.3–p13	0	13	13
24	c.856delG	p286, fsX	Frameshift	Path	–		10q26.13–26.3; 22q12.1–13.33	0	11	11
25a	c.277A > G	p.Ser93Gly	Missense	UV[h,i]	–	*6p25.3–12.3*	1p36.33–31; 3p26.3–21.31; 18q21.31–23	16	26	42
27	c.1744C > T	p.Leu582Phe	Missense	UV[i]	–	**9p24.3**	Complete Chr. 12	1	41	42
28a	c.114C > G	p.Asn38Lys	Missense	UV[i]	3	*8q23.3–24.13 9q24.3–24.1*	3p26.3–p22.3	7	11	18
29	c.112A > C	p.Asn38His	Missense	UV[i]	–	**17p13.3–q21.31** *Complete Chr. 8*	10p15.3–q21.1; 10q22.3–26.3	52	38	90
31	c.109G > A	p.Glu37Lys	Missense	UV[i]	–		3p26.3–21.31	0	16	16
32	c.2579C > T	p.Ser860Leu	Missense	UV	2		10q arm 21q arm 22q arm	0	50	50
33	c.1387–8G > T + c.1737A > G	p.? + p.Lys579Lys	Intron variant + silent	UV	–			0	0	0
34	c.2276G > A	p.Gly759Glu	Missense	UV	–	**Complete Chr. 7**	17p arm 21q21.1–22.3	44	14	58
S10				Meth	3			0	0	0
S16				Meth	–	*Complete Chr. 4*		47	0	47
S19				Meth	–	*8p23.3–p21.3,* **Complete Chr. 12**		46	0	46
S20				Meth	–	**Complete Chr. 9**	12q arm	43	29	72
S32				Meth	1			0	0	0
S39				Meth	–	*6q21–q22.31, Complete Chr. 10*		42	0	42
S43				Meth	–	**1q42.2–q44,** **6q25.3–q27**	6p25.3–q25.2, 19p13.3–p13.11	8	13	21
S51				Meth	–	**Complete Chr. 12**		41	0	41

Table 1 continued

Sample ID	DNA nucleotide change	Predicted protein change	Predicted biological type	Clinical status	Missing LP (1,2,3,4)	CNA Gain/loss	cnLOH	Segment size[g] CNA	cnLOH	Total
S69				Meth	–			0	0	0
S78				Meth	1		6p25.3–p22.1	0	10	10

[a] TNM classification of the colon tumors, http://tnm.uicc.org

[b] Mutation in a sister not in this patient

[c] Mutation in family, this person was not tested

[d] Obligate carrier

[e] Patient carried additional, *MSH6* UV [V878A]

[f] Patient carried additional *MUTYH* G382D mutation

[g] Size of segment is measured in chromosomal sub-bands

[h] Most likely a polymorphism

[i] *MLH1* promoter not hypermethylated

Abbreviations: MSI, microsatellite instability; IHC, immunohistochemistry; LP, linkage panel; CNA, copy number aberrations; cnLOH, copy neutral loss of heterozygosity; path, pathogenic; na, information not available; uns, unspecified; p.?, exact effect on the protein sequence unknown; SmB, small bowel; EC, endometrial carcinoma; UV, unclassified variant; Chr., chromosome; meth, *MLH1* promoter hypermethylation

In bold, physical loss; in italics, gain; 0, protein abrogated in tumor nuclei; +, protein expression in tumor nuclei

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were determined by hypermethylation analysis of the *MLH1* promoter using a methylation-specific MLPA assay as previously described [21].

Single nucleotide polymorphism array analysis

DNA was tested using Illumina BeadArrays and the GoldenGate assay (Illumina, San Diego, CA, USA). The GoldenGate assay was carried out according to the manufacturer's protocol with minor differences: 1 μg DNA was used as input in a multi-use activation step and subsequently dissolved in 60 μl resuspension buffer. For each sample, four SNP panels (linkage panel, LP), LP1-4, were tested together covering the genome: LP1 covers chromosomes 1–3 and 22, LP2 for chromosomes 5–9, LP3 for chromosomes 10–15 and 21, and LP4 for chromosomes 4, 16–20, X and Y. Each panel was analyzed separately on a beadarray. Due to the limited availability of archival tumor tissue some of the LPs could not be analyzed. In 13 cases one LP, and in one case two LPs could not be analyzed. Two carcinomas (cases 13 and 15,) could therefore not be analyzed for loss at *MSH2* or *MSH6*, respectively, and two for the hypermethylated *MLH1* locus (case S32 and S78, Table 1). Overall, we were able to analyze 91% of the genome in the three groups we corrected for the missing information in subsequent calculations.

We used linkage mapping panel version IV_B containing 6008 SNP markers distributed evenly over the genome with an average physical distance of 482 kb. Gene calls were extracted using GeneCall (version 6.0.7) and GTS Reports (4.0.10.0) (Illumina, San Diego, CA, USA). The software provides two quality scores: an experiment-wide gene train score (GTS) and a sample-specific gene call score (GCS).

Copy number and loss of heterozygosity (LOH)

Copy numbers were determined from the signal intensity of the individual SNPs. LOH was analyzed by comparing the genotypes from paired normal and tumor DNA. Both genomic profiles were generated with the R-package BeadArray SNP [22]. In addition, chromosome visualization of LOH was performed in Spotfire DecisionSite (Spotfire, Somerville, MA, USA) [15]. Furthermore, LOH was computed from the GCS and the GTS. LOH was called for high quality heterozygous SNPs in the normal tissue (relative gene call score (rGCS) > 0.8) that were, in the paired tumor, either homozygous or showed an rGCS/GTS ratio <0.8. In practice, regions of LOH always presented with stretches of markers showing LOH. LOH at one or two SNPs was ignored [15, 23]. Our interpretation of LOH has been verified in separate experiments with tumors using microsatellite and FISH probes (results not shown).

When both physical loss and LOH were detected at a specific region, we considered the detected LOH as an additional indication of physical loss. If no copy number change was detected, LOH was interpreted as copy neutral LOH (cnLOH).

Statistics

With a one-way ANOVA F test the amount of chromosomal aberrations in the three MSI-H groups was compared. A Scheffe-post hoc test was performed between the contrasts when the 0-hypothesis was rejected.

Results

We studied genome wide copy number changes and copy neutral LOH (cnLOH) in formalin-fixed paraffin-embedded (FFPE) tumor tissue using 6 K SNP arrays. The cohort consisted of 23 MSI-H tumors of 23 Lynch syndrome patients with pathogenic mutations eight tumors of patients with unclassified variants in *MLH1*, or *MSH2* genes. In addition, 10 sporadic MSI-H carcinomas with *MLH1* promoter hypermethylation from 10 patients were analyzed (Table 1).

Lynch syndrome cases with pathogenic MMR mutations

Immunohistochemical analysis revealed that in all carcinomas of pathogenic MMR mutation carriers the protein of the mutated gene was abrogated. In 14 of these cases both proteins of the heterodimer (MLH1/PMS2 or MSH2/MSH6) were abrogated. In six of these carcinomas (from three *MLH1* and three *PMS2* mutation carriers) also MSH6 was not expressed, this might be due to a frameshift in the C8 repeat which is located in the coding region of *MSH6* [24] (Table 1).

As expected from the literature [7, 25, 26], very few copy number aberrations were observed in the carcinomas from the carriers of pathogenic MMR mutations (Table 1). Only five of 23 (22%) MSI-H tumors presented with copy number abnormalities. Four of these cases, showed a single loss or gain of a chromosomal region. The fifth tumor presented with gain in two chromosomal regions. The chromosomes 2p, 3q, 9p, 19q and 20p were targeted in these tumors and the size of the affected segments ranged from 1 to 19 chromosomal sub-bands. Chromosome band 9p24.3 was targeted twice, in cases 7 and 17 by physical loss and gain, respectively. Physical loss of the MMR gene involved was only detected in case 10. Interestingly, in this case two different types of alterations were detected around chromosomal sub-band 2p21 harbouring the (mutated)

Table 2 Copy neutral LOH at MMR loci and mean percentage of aberrant sub-bands in cases from pathogenic MMR mutation versus MMR-UV carriers

Gene	cnLOH at locus	SRO at locus
MLH1	5/6	3p25.1–22.2
MSH2	2/6	2p25.3–14
MSH6	0/5	–
PMS2	2/5	7p22.3–13
MLH1 UV	2/5	3p26.3–21.31
MSH2 UV	0/3	–
MLH1 hypermethylation	0/8	–

Abbreviations: LOH, loss of heterozygosity; UV, unclassified variant; SRO, smallest region of overlap

MSH2 gene; physical loss adjacent to cnLOH. We designated this alteration as physical loss.

Also genome wide cnLOH was infrequent in these tumors (Table 1). However cnLOH around the locus of the mutated MMR gene was frequently observed in the 23 carcinomas from patients with pathogenic mutations. Five of six tumors from *MLH1* mutation carriers showed cnLOH at the *MLH1* locus (3p22.2) (Table 2). The extend of the LOH ranged from chromosome 3p26.3 to 3p14.1, and the smallest region of overlap (SRO) spanned 3p25.5–21.31, which encompasses *MLH1*. Two of the six *MSH2* tumors showed cnLOH of the *MSH2* locus at chromosome 2p21 (the interval of LOH ranged from 2p25.3 to 2p13.3; SRO 2p25.3–14) (Table 2). For *PMS2* mutation carriers, cnLOH was seen in two of five tumors (interval of LOH, 7p22.3–11.1; SRO, 7p22.3–13) (Fig. 1, Table 2). None of the five tumors from *MSH6* mutation carriers showed cnLOH at the *MSH6* locus (2p16, Table 2).

In addition, two of seven *MSH2* carcinomas presented with cnLOH at 6q with SRO; 6q24.3–25.2 (cases 8 and 14, Table 1). One patient with a pathogenic *PMS2* germline mutation (case 22, Table 1) presented with additional LOH of the chromosomal region 2p25.3–15 that harbours *MSH2* and *MSH6*. In this left-sided colon carcinoma, the protein expression of MLH1, MSH6 and PMS2 was abrogated and MSH2 expression was retained. *MLH1* and *MSH6* germline mutation analysis were negative.

MSI-H carcinomas with unclassified variants in MMR genes

Eight carcinomas from MMR-UV carriers were tested. Five of the eight cases showed normal positive staining of MMR proteins tested. Two *MLH1*-UV cases showed absent staining of at least MLH1, whereas one *MSH2*-UV case showed only absence of MSH6 protein. The five *MLH1*-UVs did not show promoter hypermethylation of *MLH1*.

Copy number abnormalities were detected in five of eight (62%) carcinomas from MMR-UV carriers. These carcinomas were from four *MLH1*-UV and one *MSH2*-UV carriers. Four tumors displayed a single copy number abnormality and the fifth tumor displayed two copy number abnormalities. The affected segments ranged in size from 1 to 52 chromosomal sub-bands. The copy number abnormalities affected chromosome 6p, 7, 8, 9p and 17. Chromosome 9p24.3 was affected in two of these five tumors (a gain in tumor 27 and physical loss in tumor 28a, Table 1). None of the analyzed tumors from MMR-UV carriers showed physical loss at the specific MMR gene locus involved.

CnLOH at the locus of the mutated MMR gene was found to a lesser extent than in tumors from pathogenic MMR mutation carriers (Table 2). Two of the five *MLH1*-UV carcinomas showed cnLOH at the *MLH1* locus on chromosome 3p22.2 while none of *MSH2*-UV carriers showed cnLOH at chromosome 2p21 (Table 2). Also genome wide cnLOH was limited. Five of the eight carcinomas showed cnLOH, ranging from one to three genomic regions at eight different chromosomes (Table 1).

Sporadic MSI-H carcinomas with *MLH1* promoter hypermethylation

Genome-wide profiles of copy number abnormalities and cnLOH were determined from 10 MSI-H carcinomas with *MLH1* promoter hypermethylation. Protein expression of MLH1 and PMS2 was abrogated in all 10 carcinomas as determined by immunohistochemistry. In six of the 10 (60%) sporadic MSI-H carcinomas limited copy number changes were detected. Three of these tumors exhibited one copy number abnormality and the other three displayed two changes. The affected segments ranged in size from 8 to 47 sub-bands of the genome of these carcinomas, affecting chromosome 1q, 4, 6, 8p, 9, 10 and 12. Amplification of complete chromosome 12 occurred in two cases (case S19 and S51) all additional copy number changes were unique. The locus of *MLH1* showed neither physical loss nor cnLOH in eight tumors that could be tested (Table 2). CnLOH was observed in 3 of the 10 carcinomas (30%). Two tumors showed one segment of cn LOH and the other tumor displayed two segments of cnLOH, affecting chromosomes 6, 12q and 19p (Table 1).

Comparison of three groups

We compared the average number of segments with cnLOH or copy number abnormalities detected in the carcinomas of the different groups (Table 3). The fraction of aberrant segments in each group and the distribution over the chromosomes is shown in Fig. 2. This comparison

Fig. 1 LOH view of tumors from a pathogenic *PMS2* mutation carrier ▶ generated with Spotfire DecisionSite (Spotfire, Somerville, MA). Heterozygous SNPs (upper diamonds in the figure) are dispersed over the chromosomes. These were analyzed in both tumor and corresponding normal DNA. For LOH, ≥3 SNPs in a specific region that are altered from heterozygote in normal to homozygote in tumor (lower diamonds) are scored as LOH. In practice, regions of LOH always presented with stretches of markers showing LOH. LOH at one or two SNPs was ignored. In this case LOH of *PMS2* is seen on chromosome 7 and none of the pseudogenes on chromosome 7q are affected

shows that the carcinomas of patients carrying an UV in one of the MMR genes display more aberrations (on average 2.79, range 0–4), than the carcinomas of patients with a pathogenic MMR mutation (on average 1.44, range 0–3) and the carcinomas with *MLH1* promoter hypermethylation (on average 1.32, range 0–4). The average number of aberrant segments of the three groups were compared with a one-way ANOVA test. A significant difference was found ($P = 0.045$) comparing the total number of segments per group, in a post hoc test (Scheffe test) no significant difference was revealed between the individual groups. The average size (chromosomal sub-bands) of the aberrant segments is larger in the tumors of patients carrying an UV in *MLH1* or *MSH2* (13 sub-bands) and in tumors with *MLH1* promoter hypermethylation (20 sub-bands), compared to the tumors of patients with a pathogenic MMR mutation (8 sub-bands).

Although subtle, the distribution of the types of chromosomal events—copy number aberrations versus cnLOH—is different in the carcinomas with *MLH1* promoter hypermethylation compared to the carriers of a pathogenic mutation or an UV in one of the MMR genes. Whereas in these last two groups the majority of events comprise cnLOH, copy number aberrations are more prevalent in carcinomas with *MLH1* promoter hypermethylation (Table 3). The one-way ANOVA test identified a significant difference ($P = 0.027$) between the number of cnLOH events in the three carcinoma groups; the Scheffe test assigned this result to the difference between the sporadic carcinomas with *MLH1* promoter hypermethylation and carcinomas from MMR-UV carriers ($P = 0.027$). A comparison of the percentage of chromosomal gain, loss and/or cnLOH was made for the three groups. The increase of chromosomal aberrations in carcinomas from MMR-UV carriers compared to the other two groups is again evident. The chromosomes involved and the distribution of the events over the chromosomes is different between the groups. Chromosomes 6p, 9p, 10q and 12p are affected by events in all three groups although to a different level. The suggested increase in events on chromosome 3p in the tumors from MMR-UV (Fig. 2) carriers can be explained by an unequal distribution of *MLH1* carriers (pathogenic 6/26 vs UVs 5/8) in the groups. Furthermore, one *MLH1*-UV case with cnLOH on 3p does not comprise the *MLH1* locus.
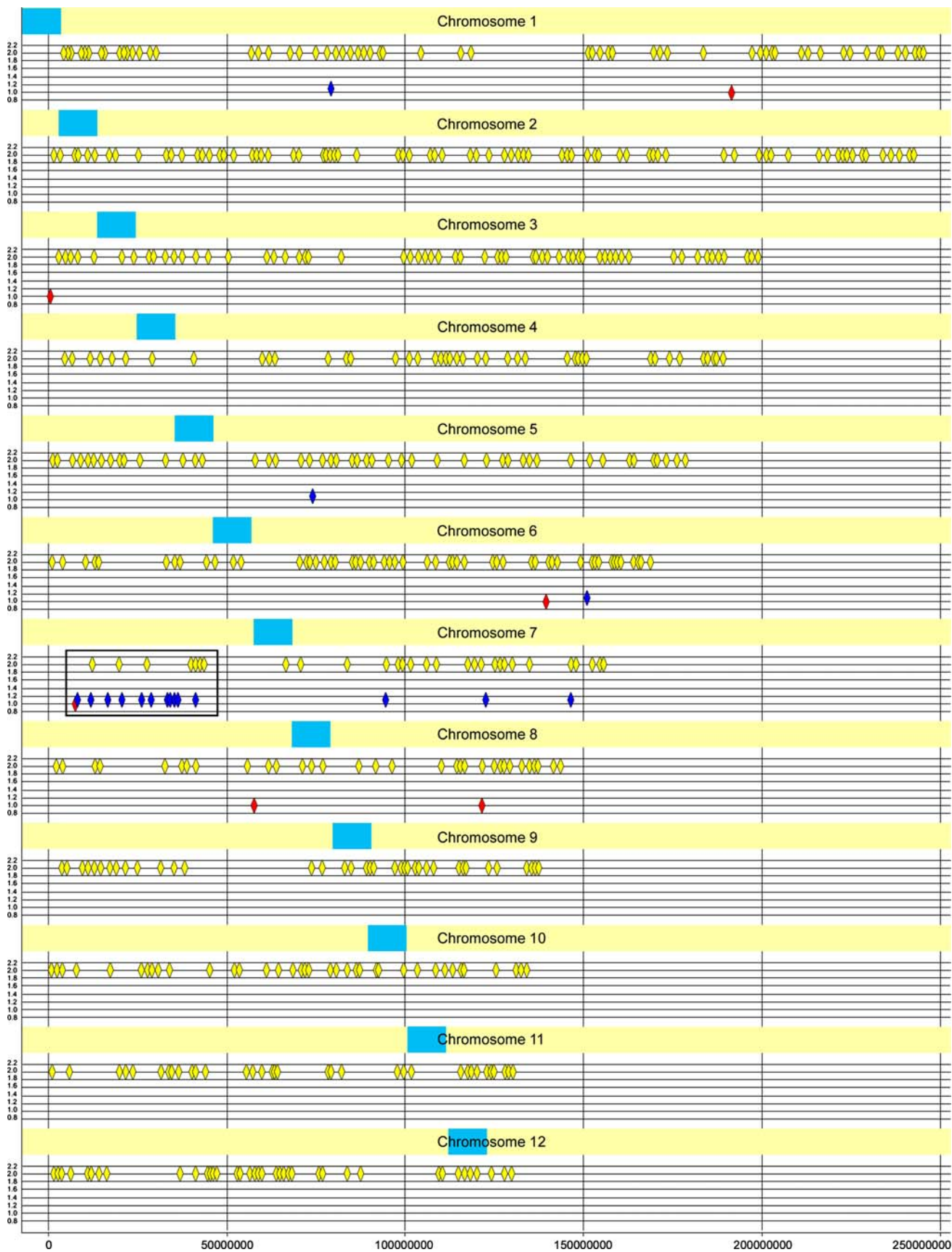
Table 3 Average number of chromosomal segments with cnLOH and genomic aberrations

	Pathogenic MMR mutation carriers	MMR-UV carriers	*MLH1* promoter hypermethylation
Gain	0.22	0.38	0.4
Loss	0.09	0.62	0.4
cnLOH	1.14	1.79	0.52
Total	1.44	2.79	1.32
Size	11.96	35.81	25.73
	N = 23	*N* = 8	*N* = 10

Abbreviations: MMR, mismatch; repair; UV, unclassified variant; cnLOH, copy neutral LOH; Size, average number of aberrant chromosomal sub-bands per carcinoma; N, number of carcinomas

Discussion

This is the first study that compares genome wide SNP array profiles of MSI-H carcinomas from MMR pathogenic mutation carriers, MMR-UV carriers and carcinomas with promoter hypermethylation of *MLH1*. With both comparative genomic hybridization (CGH) and SNP arrays, copy number information can be obtained however with SNP arrays also genome wide copy neutral LOH (cnLOH) can be studied which provide us with additional information. We used Illumina 6K SNP arrays on FFPE material and analyzed the data with the BeadArray SNP package [22].

Overall we did not detect extensive cnLOH in MSI-H carcinomas. Most of the cnLOH we found in carcinomas from pathogenic MMR mutation carriers, involved the MMR gene locus. Especially, for *MLH1* such cnLOH was seen in tumors from pathogenic mutation carriers (in five of six tumors). In the MMR-UV cases, cnLOH at the MMR locus was less frequent. In literature a varying frequency of LOH has been described on the *MLH1* and *MSH2* locus in series of pathogenic MMR mutation carriers and MMR-UV carriers. LOH at chromosome 3p has been reported in 35–85% of all tumors with a germline mutation (pathogenic as well as UVs) in *MLH1* [3, 27–34]. LOH at chromosome 2p has been described in 14–50% of all tumors with a germline *MSH2* mutation (pathogenic as well as UVs) [3, 28, 31, 35]. We detected cnLOH of *PMS2* in 40% of tumors, which to our knowledge has not been published previously. Using SNP arrays we have delineated the intervals of LOH around the affected genes. The LOH at the *PMS2* locus on chromosome 7p (SRO 7p22.3–13) points at the sensitivity of the technique in view of the existence of about 14 pseudogenes of *PMS2* [36–39] that were not targeted by the specific cnLOH of the *PMS2* locus.

Of interest is the increased number of aberrant segments in carcinomas from MMR-UV carriers compared to pathogenic MMR mutation carriers and carcinomas with *MLH1* promoter hypermethylation. Apparently, CIN is added to microsatellite instability in these MMR-UV cases during tumorigenesis. This could suggest that such additional CIN is necessary for tumorigenesis in cases with a priori weak mutator effects. Furthermore, this finding supports the observations that CIN and MIN are not mutually exclusive [9, 40–42]. With the detection of an unclassified variant in one of the MMR genes in patients that are highly suspected to be affected with Lynch syndrome, the uncertainty that a pathogenic mutation has been missed remains. We now suggest that finding a relatively increased CIN might make this less likely, as was seen in five of eight MMR-UV cases. However, the finding of MSI-H with absence of nuclear staining in cases from MMR-UV carriers does not definitively prove the pathogenicity of such UV. The five tumors from MMR-UV carriers, in which all MMR proteins tested are expressed, suggest the presence of a stable protein that is defective in MMR. It should be remembered that the staining and MSI results also depend on the nature
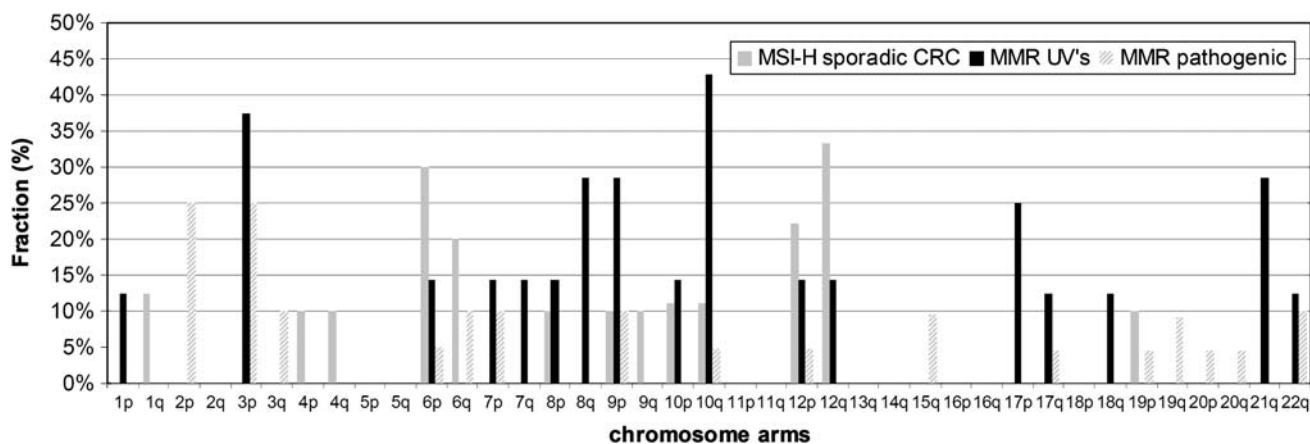

Fig. 2 Fraction of chromosomal events, per chromosome arm, in MSI-H carcinomas. The shaded bars indicate the percentage of 23 carcinomas from pathogenic mutation carriers and the black bars represent the eight carcinomas from MMR-UV carriers. The grey bars indicate the MSI-H carcinomas with hypermethylation of the *MLH1* promoter that exhibit events of chromosomal aberration of a chromosome. This percentage has been calculated for the respective chromosome arms

of the second somatic hit that occurred in the tumor. Furthermore, in series of cases with specific MMR-UVs not always the same results are obtained [19].

We see that the chromosomal segment that is targeted is larger in the tumors of patients carrying an UV in *MLH1* or *MSH2* and in tumors with *MLH1* promoter hypermethylation, compared to the tumors of patients with a pathogenic MMR mutation. Aberrations of whole chromosomes are found in, respectively, five of the eight MMR-UV carcinomas, in five of the 10 *MLH1* methylated carcinomas and only in two of the 23 MMR pathogenic carcinomas. In addition, the distribution of the types of chromosomal events—copy number aberrations versus cnLOH—is slightly different in the carcinomas with *MLH1* promoter hypermethylation compared to the carriers of a pathogenic mutation or an UV in one of the MMR genes. Whereas in these last two groups the majority of abnormalities concerns cnLOH (79% and 64%, respectively), copy number aberrations are the more prevalent abnormality seen in carcinomas with *MLH1* promoter hypermethylation (60%). In contrast to other publications we detected equal amounts of gain and physical loss of parts or whole chromosomes in the sporadic MSI-H carcinomas [9, 42]. Trautmann et al. studied 23 sporadic MSI-H carcinomas with array CGH and identified gains on chromosomes 8, 12 and 13. We also identified gain of chromosome 12 in two out of 10 carcinomas.

Moreover, we could identify several small regions with copy number changes and cnLOH that were present in more than one MSI-H tumor with a pathogenic MMR defect on chromosomes 9p24.3 and 6q24.2–25.2 respectively. These regions might harbour genes that are important for tumorigenesis. Recent association studies identified polymorphic sequences at 8q24 as associated with an increased risk for CRC. Interestingly, chromosome 9p24 was also implicated in two of these studies pointing at a role for 9p24 in carcinogenesis [43–46].

The approach we describe here appears to be an elegant way to detect (genome wide) cnLOH in MSI-H formalin fixed paraffin embedded carcinomas. Studying LOH in these type of carcinomas was often hampered due to instability of polymorphic microsatellite markers. We also suggest that the SNP array platform, as described here and applicable to FFPE tissue, may be a crucial tool in finding the genetic cause of unexplained familial colorectal cancer, since we were able to identify distinct small regions of LOH and/or copy number alterations.

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