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LOH and copy neutral LOH (cnLOH) act as alternative mechanism in sporadic colorectal cancers with chromosomal and microsatellite instability

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***Background and aims*. Tumor suppressor genes are often located in frequently deleted chromosomal regions of colorectal cancers (CRCs). In contrast to microsatellite stable (MSS) tumors, only few loss of heterozygosity (LOH) studies were performed in microsatellite instable (MSI) tumors, because MSI carcinomas are generally considered to be chromosomally stable and classical LOH studies are not feasible due to MSI. The single nucleotide polymorphism (SNP) array technique enables LOH studies also in MSI CRC. The aim of our study was to analyse tissue from MSI and MSS CRC for the existence of (frequently) deleted chromosomal regions and tumor suppressor genes located therein. *Methods and results*. We analyzed tissues from 32 sporadic CRCs and their corresponding normal mucosa (16 MSS and 16 MSI tumors) by means of 50K SNP array analysis. MSS tumors displayed chromosomal instability that resulted in multiple deleted (LOH) and amplified regions and led to the identification of *MTUS1* (8p22) as a candidate tumor suppressor gene in this region. Although the MSI tumors were chromosomally stable, we found several copy neutral LOHs (cnLOH) in the MSI tumors; these appear to be instrumental in the inactivation of the tumor suppressor gene *hMLH1* and a gene located in chromosomal region 6pter–p22. *Discussion*. Our results suggest that in addition to classical LOH, cnLOH is an important mutational event in relation to the carcinogenesis of MSS and MSI tumors, causing the inactivation of a tumor suppressor gene without copy number alteration of the respective region; this is crucial for the development of MSI tumors and for some chromosomal regions in MSS tumors.**

Introduction

Colorectal cancer (CRC) is still one of the major causes of death from malignant diseases; >945 000, new cases are diagnosed annually worldwide (1). Tumors with similar clinical and pathological features show significant prognostic differences. These differences may be, in part, due to underlying molecular genetic changes, which include chromosomal instability (CIN) and microsatellite instability (MSI). MSI is due to a defect in DNA mismatch repair, resulting in an accumulation of somatic alterations in nucleotide repeat sequences called microsatellites (2). The majority of MSI tumors are chromosomally stable, and the majority of microsatellite stable (MSS) tumors are chromosomally instable (3–5).

Tumors with CIN frequently exhibit aneuploidy (6) as well as allelic imbalance resulting in the inactivation of tumor suppressor genes (7,8). Tumor suppressor genes can be inactivated by promoter silencing as a result of methylation or dual inactivation by either mutation or a combination of mutation of one allele and loss of the retained allele [loss of heterozygosity (LOH)]. The latter is the classical definition of a CIN tumor suppressor as proposed by Knudson in 1971 (the two-hit-hypothesis). According to this hypothesis, tumor suppressor genes are inactivated by an initial mutation of the first allele followed by deletion of the second allele (9,10). Copy neutral LOH (cnLOH) or alternatively uniparental disomy (UPD) is a recently described mechanism resulting in the inactivation of tumor suppressor genes as well as the activation of oncogenes in MSS and MSI tumors (11,12). Following initial mutational inactivation or activation of one allele, the remaining wild-type allele is deleted at the same time as the mutated allele is duplicated.

We have shown that UPD seems to be involved in the activation of early-acting tumor suppressor genes in MSS (*APC*, *CDKN*2a) and MSI (*hMLH*1, *hMSH*2, *APC*, *CDKN*2a) CRC cell lines (12). UPD/cnLOH has also been found in polycythemia vera, breast and lung cancer, leukemia and many other tumors [(13–15); reviewed in ref. 16].

In contrast to MSS CRCs, only few studies of chrosomomal allelic losses (LOH/cnLOH) were performed in MSI carcinomas because they are infrequent (only 15% of sporadic CRCs) and generally considered to be chromosomally stable. Furthermore, classical LOH studies were not feasible due to the MSI and by comparative genomic hybridization and chromosomal-banding analysis cnLOH is not detected. Calhoun *et al.* (17) demonstrated that methods depending solely upon copy number analysis would theoretically identify a maximum of only 47.2% of all forms of LOH, suggesting the levels of LOH to be underestimated markedly in studies using comparative genomic hybridization. The recently developed single nucleotide polymorphism (SNP)-array technique allows the simultaneous analysis of copy number changes and genotypes in MSI and MSS tumors, thus allowing the detection of regions with copy neutral allelic losses (cnLOH) and classical LOH. We performed 50 K SNP arrays and mutational analysis of primary tumors (16 MSS tumors and 16 MSI tumors) to test the hypothesis that tumor suppressor genes/oncogenes are frequently inactivated/activated by cnLOH in MSS and MSI CRC.

Material and methods

Patient characteristics and DNA extraction

Between July 1999 and March 2004, a total of 165 CRC tissues and corresponding normal mucosa were collected during operations performed in the Department of Surgery, University of Wuerzburg. The collection was approved by the local Ethics Committee. Specimens containing at least 50% neoplastic cells (assessed by histological examination) were snap frozen in liquid nitrogen and subsequently stored at −80°C. In all cases, fresh specimens of appending normal colon mucosa were also collected and used as matching controls. DNA was extracted from tumors and matching normal sample tissues using a QIAGEN DNeasy tissue kit (QIAGEN, Hilden, Germany). The extracted DNA was diluted to a concentration of 100 ng/ml.

MSI analysis

The National Cancer Institute microsatellite panel (BAT25, BAT26, D2S123, D5S346 and D17S250) was used for MSI analysis (18). For polymerase chain

Abbreviations: CIN, chromosomal instability; CRC, colorectal cancer; MSS, microsatellite stable; LOH, loss of heterozygosity; MSI, microsatellite instability; SNP, single nucleotide polymorphism; PCR, polymerase chain reaction; UPD, uniparental disomy.

reaction (PCR), one of the primers from each primer pair was labeled with a fluorescent dye (cy-5, cy5.5; TIB MoLBioL, Berlin, Germany) that enables detection of the resulting PCR product. PCR was performed in a 25 µl reaction mixture, containing 50 nM primers, 200 µM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$ in ×1 PCR buffer, 50 ng template DNA and 0.4 U *Taq* polymerase. An initial 3 min denaturation at 96°C was followed by 33 cycles of 96°C for 20 s, 58°C for 20 s and 72°C for 30 s. Electrophoretic separation of the amplified PCR products was performed using a CEQ8000 (Beckman Coulter, Krefeld, Germany). An external size-standard facilitated size determination of the PCR products. MSI-L (low MSI) was defined as occurring when one marker out of five (BAT25, BAT26, D2S123, D5S346 and D17S250) was unstable. MSI-H (high MSI) was defined as occurring when more than two markers exhibited instability.

Immunohistochemistry

To assess the expression status of the mismatch repair proteins MLH1 (MutL protein homolog 1), MSH2 (mutS homolog 2), MTUS1 (mitochondrial tumor suppressor 1), NOL7 (nucleolar protein 7), ID4 (inhibitor of DNA binding 4), SOX4 (sex determining region Y) and E2F3 (E2F transcription factor 3) in tumor and normal tissue, immunophenotyping was performed on sections of formalin-fixed and paraffin-embedded tissue. The antibodies MLH1 (dilution 1:200; BD Bioscience, Heidelberg, Germany), MSH2 (dilution 1:40; Calbiochem, Schwalbach, Germany), MTUS1 (dilution 1:500; Abnova, Heidelberg, Germany), NOL7 (dilution 1:500; Abnova), ID4 (dilution 1:100; Abcam, Heidelberg, Germany), SOX4 (dilution 1:100; Abcam) and ESF3 (dilution 1:100; Abcam) were applied using a modified streptavidin–peroxidase antiperoxidase technique (19). In the case of a mismatch repair protein loss, no nuclear staining would be found in the tumor tissue, unlike the normal tissue.

Mutational analysis

Control fragments for use in PCR–single-strand conformation polymorphism analysis were cloned from human carcinoma cell lines with known mutations or were constructed by site-directed mutagenesis. The following mutations were used as positive controls: codon 600 (GTG→GAG) in the *B-RAF* gene; codon 33 (TCT→TAT), codon 41 (ACC→GCC and ACC→ATC), codon 45 (TCT→CCT and TCT→TTT) and *del* codon 45 (deletion of the complete codon) in *CTNNB1*. Mutational analyses of *B-RAF*, *CTNNB1* and *APC* genes were performed as described earlier (20). Briefly, for PCR–single-strand conformation polymorphism mutational analysis of *B-RAF* and *CTNNB1*, a sample of 20 ng genomic DNA was amplified by conventional PCR with sequence-specific primers flanking codons 582–620 and 6–76, respectively. The amplicons were purified from a 2% agarose gel. Electrophoresis of 4 µl of the denatured product was performed using a 16 × 20 cm non-denaturing polyacrylamide gel in ×1 TBE (90 mM Tris base, 90 mM boric acid, 2 mM ethylenediaminetetraacetic acid; pH 8,0) with 20 mmol/l *N*-(2-hydroxyethyl)-piperazine-1-*N′*-(2-ethanesulfonic acid). In order to detect the *B-RAF* mutation, a 10% polyacrylamide gel was run at 18°C with 300 vol for 4.5 h. In the case of the *CTNNB1* mutation analysis, a 13% gel was used at 26°C with 250 vol for 5 h and an 8% gel at 21°C with 200 vol for 5 h. Gels were silver stained and any shifted bands were excised, reamplified, purified and sequenced. For the mutational analysis of the *APC* gene all exons and exon–intron boundaries were amplified (supplementary Table 1 is available at *Carcinogenesis* Online). PCR was performed in 25 µl reactions containing 2.5 µl of ×10 *Taq* polymerase buffer, a 0.2 mM concentration of each deoxynucleoside triphosphate, a 0.2 µM concentration of each primer, 1 U of *Taq* DNA polymerase and 20 ng of template DNA. DNA sequencing was performed on PCR products purified with ExoSAP-IT (USB Europe, Staufen, Germany). Sequence reactions were carried out using Quick-Start Dye Terminator Cycle Sequencing kit (Beckman Coulter) and analyzed on a CEQ8000 (Beckman Coulter) according to the manufacturer's protocol. Identification of somatic mutations was followed by amplification and sequencing of corresponding parts of the *APC* gene using DNA extracted from normal tissue to exclude germ line mutations.

SNP array analysis

SNP array analysis was performed according to standard protocols (Affymetrix, München, Germany; www.Affymetrix.com). Briefly, a 250 ng sample of either amplified DNA or unamplified DNA was digested with XbaI and ligated to an XbaI adapter before subsequent PCR amplification using AmpliTaq Gold (Applied Biosystems, Foster City, CA). To obtain sufficient PCR products, four 100 µl PCRs were set up for each XbaI adapter-ligated DNA sample. The PCR products were pooled, purified, fragmented with DNase I and visualized on a 4% TBE agarose gel to confirm that the sizes ranged from 50 to 100 bp. Subsequently, the fragmented PCR products were end labeled with biotin and hybridized to the array. Detection was performed using the Affymetrix Fluidics station 450 and the GeneChip® scanner 3000. Scanned data files were generated using Affymetrix GeneChip Operating Software, version 1.2, and genotype calls were determined automatically using GeneChip DNA Analysis Software, version 3.0.

Genotypes and probe intensities derived from normal tissue and cancer DNA were loaded into the dChip software package (http://www.dchip.org/) for LOH and copy number analysis, as described previously (21). Briefly, the LOH call was assigned, for a pair of tumor and matched normal tissue samples, as LOH or allelic imbalance (heterozygosity in the normal tissue, homozygosity in the tumor), retention of heterozygosity (heterozygosity in both the normal tissue and the tumor) or non-informative (homozygosity in the normal tissue). The hidden Markov model from dChip was used to infer regional LOH with the LOH call threshold was set at 0.5. For calculating the copy number, data were normalized to a baseline array with median signal intensity, using the invariant set normalization method. The model-based (perfect match/mismatch) method was used to obtain the signal values after normalization. Normalized intensities in matched normal tissue DNA samples were used as the reference set to calculate the copy number of each marker in the tumor samples. The hidden Markov model was used to determine copy number change along each chromosome.

Results

Mutational and SNP array analysis of 16 MSS and 16 MSI CRC tissues and corresponding normal mucosa

MSI analysis and immunohistochemistry of MLH1 and MSH2 enabled identification of 16 patients with an MSI-H tumor. MLH1 was inactivated in 13 of the 16 tumors, MSH2 in 2 of the 16 and in 1 tumor neither MLH1 nor MSH2 was inactivated. The latter may be a case of dysfunctional protein that has retained antigenicity. Of the remaining 149 tumors without MSI-H, we randomly chose 16 tumors for further characterization. The resulting 32 tumors were further characterized by mutational analysis of the *APC*, *B-RAF* and *CTNNB1* genes. Data regarding the patients, the tumors and the mutational profile are summarized in Tables I and II. Although *B-RAF*-mutations were almost exclusively found in the MSI-H tumors, *APC*-mutations were predominantly found in the MSS tumors; *CTNNB1* mutations were rarely found in both subgroups. *B-RAF*-mutant tumors also generally display inactivation of hMLH1, and due to its strong association with silencing of hMLH1, *B-RAF* mutation can be considered a surrogate marker for promoter methylation of hMLH1 in sporadic MSI cancers.

After mutational analysis, a 50 K SNP array analysis of the 32 tumors was performed. SNP array analysis using the 'dchip'-Software allowed the identification of monoallelic chromosomal regions in MSS (Figure 1A) and MSI cell lines (Figure 2A) and facilitated the determination of their appropriate copy numbers (Figures 1B and 2B). Monoallelic regions with reduced copy numbers represent regions with classical deletions (LOH). Monoallelic regions without changes in the copy number represent regions with copy neutral LOH (cnLOH) (Figures 1 and 2; Table II).

Monoallelic chromosomal fragments in MSS colorectal carcinomas

Monoallelic regions were frequently (>50%) found in the 5q22.1–q22.3, 8pter–8p12, 17p13.2–13.1 and 18q11.2–qter regions (Figure 1, Table II). In these regions, genes associated with colon carcinogenesis could be assigned to *APC* (5q21–22), *TP53* (17p13.1), and *SMAD4* (18q21.1). More than half of the tumor regions (five of eight), displaying loss of heterozgosity in 5q22.1–q22.3, exhibited no concomitant copy number alterations (cnLOH). Mutations in the APC gene were found in all tumors displaying either LOH with concomitant deletion or cnLOH in 5q22.1–q22.3. Twelve of 16 tumors exhibited allelic loss in 17p13.2–13.1 and 30% of these allelic losses were copy neutral events. In contrast to 5q22.1–q22.3 and 17p13.2–13.1, 8pter–p12 and 18q11.2–qter were frequently lost, resulting in a monoallelic region with a reduced copy number. *SMAD4* is located in 18q11.2–qter, but so far no tumor suppressor gene associated with colon cancer carcinogenesis has been identified in the 8p region. *MTUS1* ('mitochondrial tumor suppressor 1') is a recently described candidate tumor suppressor gene, located at 8p22. In order to determine the relevance of *MTUS1* as a potential target of this deletion, we performed an immunohistochemical analysis of the *MTUS1* expression

Table I. Characterization of 32 tumors by MSI analysis, immunohistochemistry (IHC) of MLH1 and MSH2 and mutational analysis (Mut) of the *B-RAF*, *CTNNB1*(β-*Catenin*) and *APC* genes

ID	Sex	Age	Stage (UICC)	Grading	MSI	MLH1 (IHC)	MSH2 (IHC)	BRAF (Mut)	CTNNB1 (Mut)	APC (Mut)
MSS tumors										
21	F	79	4	G2	—	pos	pos	WT	WT	WT
29	M	56	2	G2	—	pos	pos	WT	WT	WT
33	M	44	4	G2	—	pos	pos	WT	WT	c.3927 del AAGA
51	M	77	3	G2	—	pos	pos	WT	WT	c.3908 del AA
123	M	67	4		—	pos	pos	WT	WT	WT
127	M	72	2	G2	—	pos	pos	WT	WT	c.847 C>T
132	M	71	3	G2	—	pos	pos	WT	WT	c.1690 C>T
143	M	73	1	G3	—	pos	pos	WT	WT	c.4025 insT
167	F	66	4	G3	—	pos	pos	c.1799 T>A	WT	WT
168	F	72	4	G3	—	pos	pos	WT	WT	IVS10-2 A>G
169	F	55	4	G3	—	pos	pos	WT	WT	WT
170	M	64	2	G2	—	pos	pos	WT	WT	c.637C>T
172	F	63	4	G2	—	pos	pos	WT	WT	c.4057 G>T
179	M	63	4	G2	—	pos	pos	WT	WT	WT
182	F	64	2	G2	—	pos	pos	WT	c.133 T>C	c.4284 delA
189	M	74	3	G2	—	pos	pos	WT	WT	c.4416 ins 98 bp
MSI tumors										
15	F	66	1	G2	high	neg	pos	WT	c.133 T>G	WT
25	F	85	3	G3	high	neg	pos	c.1799 T>A	WT	c.3779 A>G
55	M	38	1	G2	high	pos	neg	WT	WT	WT
59	M	60	1	G2	high	neg	pos	WT	WT	WT
64	F	86	2	G3	high	neg	pos	c.1799 T>A	WT	WT
75	F	76	2	G2	high	neg	pos	c.1799 T>A	WT	WT
99	F	30	2	G2	high	pos	neg	WT	WT	c.4666 ins A
104	F	72	2	G3	high	neg	pos	WT	WT	WT
109	F	83	4	G3	high	neg	pos	c.1799 T>A	WT	WT
122	F	74	1	G2	high	neg	pos	c.1799 T>A	WT	WT
126	M	62	1	G2	high	neg	pos	WT	WT	c.4660 G>A
131	F	64	1	G2	high	pos	pos	c.1799 T>A	WT	WT
181	M	67	2	G3	high	neg	pos	WT	c.133 T>C	WT
186	F	73	2	G2	high	neg	pos	c.1799 T>A	WT	WT
32	F	76	3	G3	high	neg	pos	c.1799 T>A	WT	WT
98	M	80	2	G2	high	neg	pos	c.1799 T>A	WT	WT

UICC—Union for International Cancer Control (tumor staging), ('—', no instability; 'high', MSI-high). Expression analysis of MLH1 and MSH2 was performed by IHC ('pos', retained staining in tumor tissue; 'neg', absent staining in tumor tissue). BRAF, CTNNB1 and APC were analyzed by mutational analysis (Mut) ('WT', wild-type/No mutation found, 'CdXXX Y->Z'—mutation in Codon XXX base X to Z, 'Ins Y CdXXX'—insertion of base Y in Codon XXX).

Table II. Analysis of allelic loss and minimal loss regions in 32 tumors by 50 K SNP array analysis

ID	chromosome band	Start	End	Size	LOH	cnLOH	Allelic loss	Gene
MSS CRC tissues (n = 16)								
1	5q22.1–q22.3	110.827.000	113.651.000	2.824.000	3/16	5/16	8/16	APC
2	8pter–p12	0	32.218.000	32.218.000	10/16	none	10/16	???
3	17p13.2–p13.1	5.762.000	10.559.000	4.797.000	8/16	4/16	12/16	p53
4	18q11.2–qter	22.361.000	76.900.000	54.539.000	13/16	none	13/16	SMAD4
MSI CRC tissues (n = 16)								
1	6pter–p22.2	0	25.446.000	25.446.000	none	4/16	4/16	???
2	3pter–p21.31	0	46.513.000	46.513.000	none	3/16	3/16	MLH1

Monoallelic regions with reduced copy numbers represent regions with classical deletions (LOH). Monoallelic regions without changes in the copy number represent regions with copy neutral LOH (cnLOH). Allelic loss is defined as LOH or cnLOH. In the table, frequently affected regions are shown (>50% for MSS tumors, >1 for MSI tumors). Start, end and size defines the minimal lost chromosomal region found in all affected tumors. Gene–gene that is localized in the minimal loss region.

on 10 of the 16 tumors with a deletion in 8pter–p12 and corresponding normal mucosa as reference tissue (supplementary Figure 1 is available at *Carcinogenesis* Online). A complete absence of staining was found in 4 of the 10 tumors, two others exhibited significant reduction in the staining intensity, two exhibited the same level of staining of *MTUS1* as in the reference tissue and two could not be evaluated because there was insufficient material. A sequencing analysis of the three tumors with a complete absence of staining was performed, but no mutation in the *MTUS1* gene could be identified.

Monoallelic chromosomal fragments in MSI colorectal carcinomas

In comparison with MSS tumors, fewer chromosomal alterations were found in MSI tumors (Figure 1, Table II). Monoallelic regions in MSI tumors were always reduplicated after an initial deletion, resulting in copy neutral LOH. Most frequently altered were the 6pter–p22.2 (4/16) and 3pter–p21.31 (3/16) regions (Table III). Unique allelic losses were found in chromosomal regions 4q24–qter, Chromosome 9, 13q12.11–qter and 15q12–qter, 19p (Figure 2). In 3pter–p21.31, the hMLH1 gene is localized (3p21.3). The absence of staining of *hMLH1*

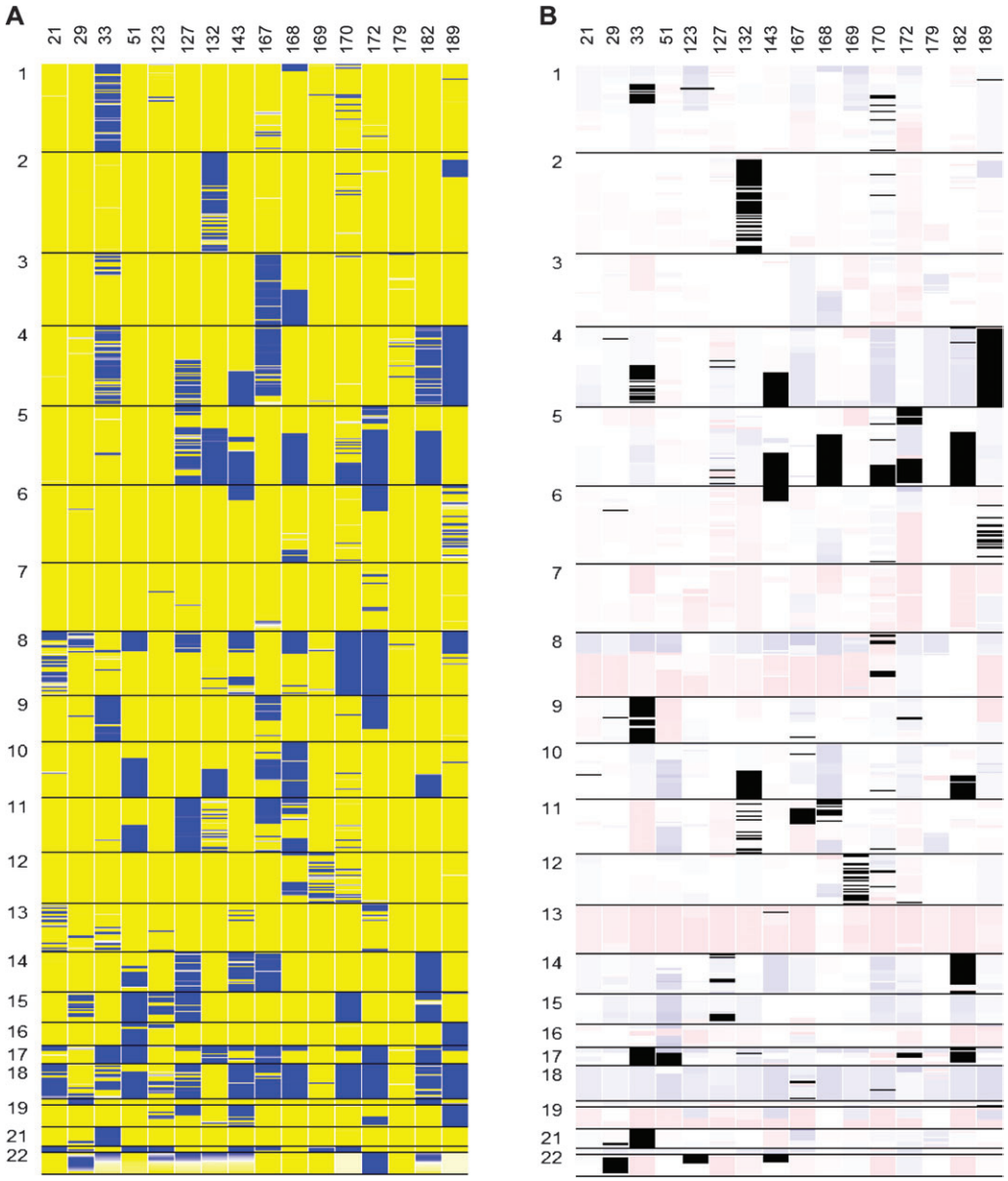

Fig. 1. SNP array (**A**) and copy number (**B**) analysis of MSS CRCs. Dark blue bands (A) represent allelic losses (LOH and cnLOH). In some chromosomal regions, allelic losses are incomplete (interrupted by non-allelic loss regions) and in some other regions, there are only scattered bands. This can be explained by a contamination of normal mucosa and/or different clones. In (B) a reduction of copy number is shown in blue color, a gain in red. Regions displaying cnLOH are represented by black bands and should match with the allelic loss regions in (A). In some chromosomal regions (e.g. 8p-tumor 170 and 18q-tumor 179), there are only scattered bands that do not match allelic losses in (A), so that these bands were not counted as cnLOH.

occurred in all tumors with allelic loss in 3pter–p21.31. A tumor suppressor gene in 6pter–p22.2 has not yet been described for colorectal carcinomas. We chose *ID4* (6p22.3), SOX4 (6p22.2), *NOL7* (6p23) und *E2F3* (6p22.3) as candidate genes in this region (because of potential relevance for CRC and available antibodies). A cnLOH was found in 4 of the 16 MSI tumors. NOL7, SOX4 and ID4 exhibited no reduction in the staining intensity in tumors and we found only one tumor in which staining was absent for E2F3 (supplementary Figure 2 is available at *Carcinogenesis* Online).

Discussion

We found recurrent cnLOH and LOH regions in 5q22.1–q22.3, 17p13.2–13.1, 18q11.2–qter and 8pter–p12 in MSS carcinomas. In 5q22.1–q22.3 and 17p13.2–13.1, either LOH or cnLOH is frequently found (8 and 12 of 15 samples, respectively). There is a trend for reduplication of the 5q region resulting in cnLOH in five of the eight cases examined, so that the cancer cells may have a growth advantage if the copy number in 5q22.1–q22.3 is preserved. Whereas tumor suppressor genes located in 5q22.1–q22.3, 17p13.2–13.1 and 18q11.2–qter are already known (*APC*, *TP53*, *SMAD4*), no tumor suppressor gene that is associated with colon cancer carcinogenesis has yet been identified in the 8pter–p12 region. *MTUS1* (mitochondrial tumor suppressor 1) is a recently described candidate tumor suppressor gene, located at 8p22 (22). In order to test the relevance of MTUS1 as a potential target gene of this alteration, we performed immunophenotyping (supplementary Figure 1 is available at *Carcinogenesis* Online) of the region in 10 of 15 tumors with a deletion in 8pter–p12 and recorded the absence or marked reduction of staining of *MTUS1* in six of these, associated with an allelic loss of 8pter–p12 in these tumors. In a recent article Zuern *et al.* (23) also demonstrated a downregulation of MTUS1 expression in colon cancer tissues and

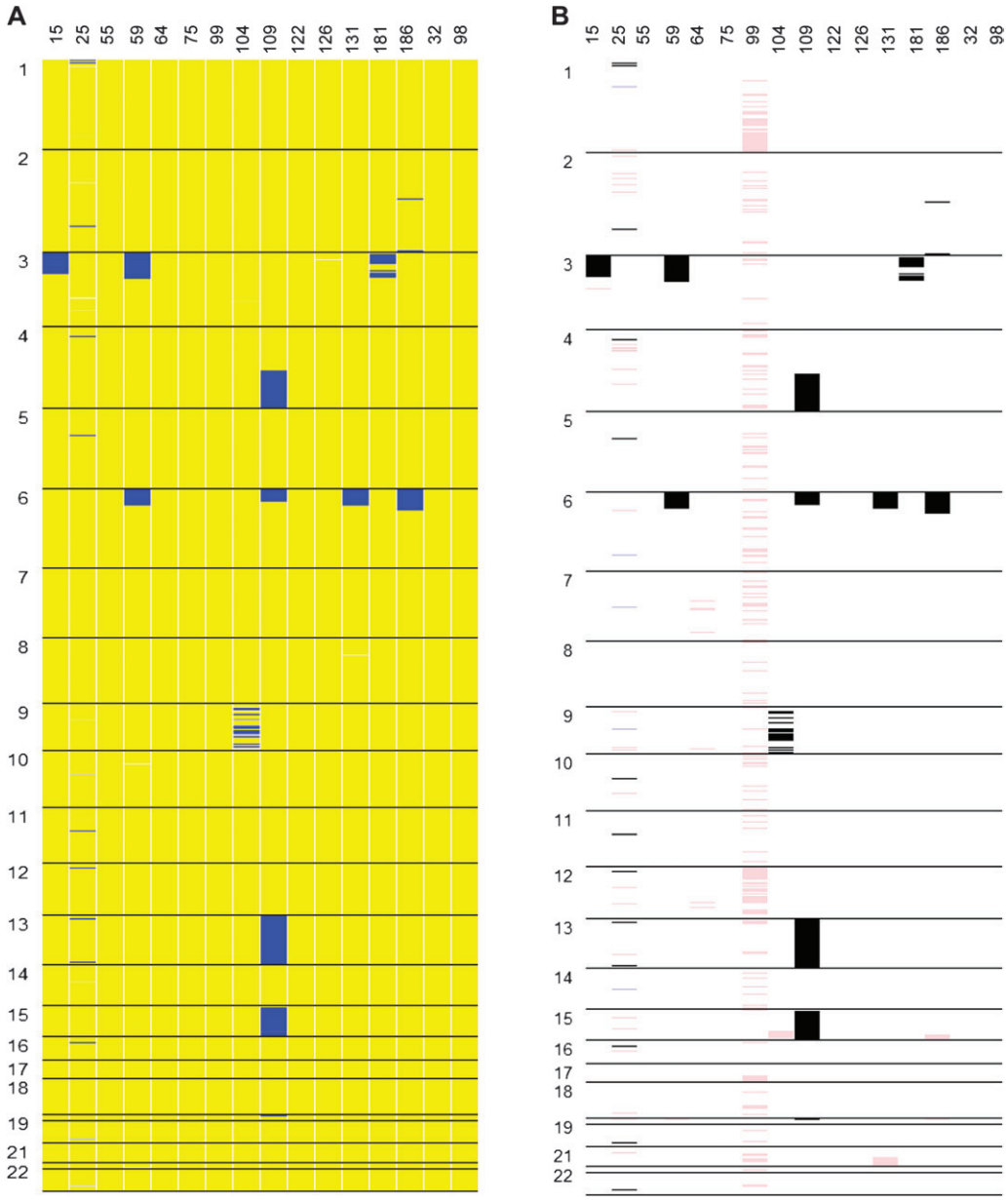

Fig. 2. SNP array (**A**) and copy number (**B**) analysis of MSI CRCs. Dark blue bands (A) represent allelic losses (LOH and cnLOH). In some chromosomal regions, allelic losses are incomplete (interrupted by non-allelic loss regions) and in some other regions, there are only scattered bands. This can be explained by a contamination of normal mucosa and/or different clones. In (B) a reduction of copy number is shown in blue color, a gain in red. In tumor 99, there seem to be a gain of almost all chromosomal regions that can be interpreted as variation of signal intensity in this specific array.

detected no mutations of MTUS1 in the coding sequence or the predicted promoter region in cancer tissues. Furthermore, they found no difference of CpG methylation in the predicted promotor region, but an altered CpNpG methylation. Thus, while further analysis of the mechanisms of downregulation is required, *MTUS1* seems to be an interesting candidate tumor suppressor gene in the 8p22 region.

In MSI carcinomas, frequent allelic losses were found in 3pter–p21.31 (3/16) and, even more frequently, in 6pter–p22.2 (4/16). In 3pter–p21.31, *hMLH1* is localized (3p21.3). In all tumors with cnLOH of 3pter–p21.31, we recorded the absence of staining of *hMLH1* during immunophenotyping, suggesting that cnLOH plays a role in the inactivation of *hMLH1*. Nevertheless, no cnLOH was found in 10 of the 13 tumors without *hMLH1* staining, so that mechanisms other than cnLOH must be involved in the inactivation of *hMLH1*. Frequent allelic losses in 6pter–p22.2 in MSI carcinomas have also been described by van Puijenbroek *et al.* (24). To date, no tumor suppressor gene has been identified in 6pter–p22.2. The affected region spans 25 Mb and contains 106 genes. In this study, we analyzed four genes that were interesting candidate tumor suppressors [*NOL7* (13723538-13729106; 6p23), *ID4* (19846839-20047640; 6p22.2), *SOX4* (21701951-21706828; 6p22.2) and *E2F3* (20510116-20601924; 6p22.2)]. *NOL7*, *SOX4*, *ID4* and *E2F3* showed no alteration in tumors. Consequently, these genes seem to be of no or little relevance for tumorigenesis, even though they could be truncated or altered in another way that could not be identified by immunohistochemistry. There are few reports of an involvement of the 6p chromosomal region in colorectal carcinogenesis. Honchel *et al.* (25) performed microsatellite analysis of 64 CRC tissue samples and found frequent allelic losses distal of the marker D6S271 (6p21.1). Studies with a larger series of MSI carcinomas have to be performed in order to define a more precise minimal deleted/altered region in 6pter–p22.2.

Table III. Monoallelic chromosomal fragments in MSI and MSS colorectal carcinomas and corresponding genes inactivated by LOH/cnLOH

MSS tumors							
ID	8pter–p12	MTUS1 (IHC)	5q22.1–q22.3	APC (Mut)			
21	LOH	na					
29	LOH	neg					
33			LOH	c.3927 del AAAGA			
51	LOH	↓↓					
123							
127	LOH	na	LOH	c.847C>T			
132			cnLOH	c.690C>T			
143	LOH	neg	cnLOH	c.4025insT			
167							
168	LOH	pos	cnLOH	IVS10-2 A>G			
169							
170	LOH	neg	cnLOH	c.637C>T			
172	LOH	↓↓	LOH	c.4057G>T			
179	LOH	neg					
182			cnLOH	c.4284delA			
189	LOH	pos					
MSI tumors							
ID	3pter–p21.31	MLH1 (IHC)	6pter–p22.2	NOL7 (IHC)	ID4 (IHC)	E2F3 (IHC)	SOX4 (IHC)
15	cnLOH	neg					
25		neg					
55		pos					
59	cnLOH	neg	cnLOH	pos	pos	pos	pos
64		neg					
75		neg					
99		pos					
104		neg					
109		neg	cnLOH	pos	pos	pos	pos
122		neg					
126		neg					
131		pos	cnLOH	pos	pos	pos	pos
181	cnLOH	neg					
186		neg	cnLOH	pos	pos	pos	pos
32		neg					
98		neg					

In MSS tumors, a chromosomal loss of the region 5q22.1–22.3 is associated with a mutation of the APC gene. Expression of MTUS1 (8p22) is downregulated or absent in 6/10 tumors with a loss of 8pter–8p12 (IHC analysis: 'pos', retained staining in tumor tissue; 'neg', absent staining in tumor tissue; 'na' - not analysed because of insufficient material). In MSI tumors, a loss of the region 3pter–p21.31 was associated with an absent expression of MLH1 in 3/3 affected tumors. IHC analysis of NOL7, SOX4 and ID4 exhibited no reduction in the staining intensity in tumors and we found only one tumor in which staining was absent for E2F3.

In contrast to our previous study (12), we found cnLOH-regions not only limited to regions where early-acting tumor suppressor genes (MLH1, APC) are located, but also in the genomic region of TP53, which is a late acting gene in the adenoma-carcinoma sequence of MSS colorectal carcinomas. Consequently cnLOH seems not to be limited to early tumor development but is a mutational mechanism affecting multiple regions in all tumor stages and tumor subgroups (MSI) and conferring a growth advantage by creating a stable copy number in the affected region.

There are three other studies that deal with SNP array analysis and CRC. Andersen *et al.* (11) examined 15 laser-microdissected CRC tissues and the corresponding normal mucosa. All carcinomas were sporadic and MSS. They also found commonly affected regions (>25% of the tumors analyzed) in 8q, 5q, 17p and 18q. In addition, they identified 3p, 13q, 14q, 15q, 20p and 22q as recurrently altered regions. In a similar approach, Kurashina *et al.* (26) analyzed 94 colorectal carcinomas. They found recurrent copy number abnormalities in CRC associated with chromosomes 7, 8, 13, 18 and 20, and recurrent deletions in chromosomes 1p, 4q, 5q, 8p, 11q, 14q, 15q, 17p, 18 and 22. In contrast to our study, they found frequent UPD not only in 5q and 17p but also in chromosomes 8p and 18q. Van Puijenbroek *et al.* (24), using 6 K SNP arrays, analyzed 10 sporadic MSI-H carcinomas with hMLH1 promoter hypermethylation from 10 patients. In 6 of the 10 sporadic MSI-H carcinomas, few small 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 chromosomes 1q, 4, 6, 8p, 9, 10 and 12. Amplification of the entire chromosome 12 occurred in two cases; all additional copy number changes were unique. The genomic locus of *MLH*1 showed neither physical loss nor cnLOH in eight tumors that could be tested. CnLOH was observed in 3 of the 10 carcinomas (30%). Two tumors exhibited one segment of cnLOH (12q, 6p25.3–p22.1) and the other tumor displayed two segments (6p25.3–q25.2, 19p13.3–p13.11). The region most frequently affected was 6pter–p22.2, which corresponds exactly to the minimal deleted region identified in our study.

Conclusion

In this study, we found allelic losses in MSS and MSI tumors. In MSS tumors, more losses than in MSI tumor were identified. Nethertheless, the results suggest that cnLOH is an important mechanism involved in the carcinogenesis of MSS and MSI tumors, enabling the inactivation of a tumor suppressor gene without copy number alteration of the respective region; this may be crucial for MSI tumors (3pter–p21.31—*MLH1*) and for some chromosomal regions in MSS tumors (5q22.1–q22.3—*APC* and 17p13.2–13.1—*TP53*). SNP array analysis resulted in the identification of *MTUS1* (8p22) as a candidate tumor suppressor gene in the frequently lost 8pter–p12 region in MSS colorectal carcinomas. In addition, the chromosomal region 6pter–p22.2

was identified as a frequently altered region in MSI carcinomas. In further studies, a greater number of MSI tumors need to be analyzed in order to narrow the minimal deleted region in chromosome 6p to facilitate identification of the tumor suppressor gene located there. A mutational analysis of tumors exhibiting loss of *MTUS1* expression is required and functional studies need to be performed to confirm that *MTUS1* is a tumor suppressor gene with relevance for colon cancer carcinogenesis.

Supplementary material

Supplementary Table 1 and Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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