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ORIGINAL ARTICLE

Array CGH identifies distinct DNA copy number profiles of oncogenes and tumor suppressor genes in chromosomal- and microsatellite-unstable sporadic colorectal carcinomas

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Abstract DNA copy number changes represent molecular fingerprints of solid tumors and are as such relevant for better understanding of tumor development and progression. In this study, we applied genome-wide array comparative genomic hybridization (aCGH) to identify gene-specific DNA copy number changes in chromosomal (CIN)- and microsatellite (MIN)-unstable sporadic colorectal cancers (sCRC). Genomic DNA was extracted from microdissected, matching normal colorectal epithelium and invasive tumor cells of formalin-fixed and paraffin-embedded tissues of 22 cases with colorectal cancer (CIN=11, MIN=11). DNA copy number changes were determined by aCGH for 287 target sequences in tumor cell DNAs, using pooled normal DNAs as reference. aCGH data of tumor cell DNAs was confirmed by fluorescence in situ hybridization (FISH) for three genes on serial tissues as those used for aCGH. aCGH revealed DNA copy number changes previously described by metaphase CGH (gains 7, 8q, 13q, and 20q; losses 8p, 15q, 18q, and 17p). However, chromosomal regions 20q, 13q, 7, and 17p were preferentially altered in CIN-type tumors and included DNA amplifications of eight genes on chromosome 20q (TOP1, AIB1, MYBL2, CAS, PTPN1, STK15, ZNF217, and CYP24), two genes on chromosome 13q (BRCA2 and D13S25), and three genes on chromosome 7 (IL6, CYLN2, and MET) as well as DNA deletions

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The main research interests of their molecular tumorpathology group focuses on the biology of solid (gastrointestinal) tumors and hematologic neoplasms, in particular the basic molecular mechanisms of tumor development and progression and the evaluation of novel applications of molecular pathology in a clinically relevant setting, such as therapy prediction.

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The main research interests of their molecular tumorpathology group focuses on the biology of solid (gastrointestinal) tumors and hematologic neoplasms, in particular the basic molecular mechanisms of tumor development and progression and the evaluation of novel applications of molecular pathology in a clinically relevant setting, such as therapy prediction.

of two genes on chromosome 17p (HIC1 and LLGL1). Finally, additional CIN-tumor-associated DNA amplifications were identified for EXT1 (8q24.11) and MYC (8q24.12) as well as DNA deletions for MAP2K5 (15q23) and LAMA3 (18q11.2). In contrast, distinct MIN-tumor-associated DNA amplifications were detected for E2F5 (8p22–q21.3), GARP (11q13.5–q14), ATM (11q22.3), KAL (Xp22.3), and XIST (Xq13.2) as well as DNA deletions for RAF1 (3p25), DCC (18q21.3), and KEN (21q tel). aCGH revealed distinct DNA copy number changes of oncogenes and tumor suppressor genes in CIN- and MIN-type sporadic colorectal carcinomas. The identified candidate genes are likely to have distinct functional roles in the carcinogenesis and progression of CIN- and MIN-type sporadic CRCs and may be involved in the differential response of CIN- and MIN-type tumor cells to (adjuvant) therapy, such as 5-fluorouracil.

Keywords Colorectal cancer · Genetic instability · Oncogenes · Tumor suppressor genes · Array CGH

Introduction

Alterations of genomic DNA at the level of whole chromosomal regions or individual genes represent molecular fingerprints of solid tumors [1, 2]. Specific patterns of DNA copy number gains/losses of entire chromosomal regions and/or target gene-specific amplifications/deletions were identified for individual tumors and their precursor lesions [3–5], including colorectal cancer [6, 7].

The development of sporadic colorectal cancer (sCRC) is characterized by specific genetic alterations, which accompany the tumor's specific macroscopic and histologic changes and hence also influence individual tumor progression [8–10]. At the DNA level, these genetic alterations were investigated by metaphase comparative genomic hybridization (mCGH) and fluorescence in situ hybridization (FISH), resulting in the definition of a specific pattern of DNA gains at 7, 8q, 13q, and 20q as well as DNA losses at 5q, 8p, 17p, and 18q [1–16]. In addition, individual oncogenes and tumor suppressor genes, located within these chromosomal regions, were identified and shown to play a major role in colorectal carcinogenesis, such as APC at 5q, DCC at 18q, and MYC at 8q. However, the detailed profile of gene-specific changes and its contribution to sporadic colorectal carcinogenesis and tumor progression is still unknown. This is complicated by the fact that the development of sCRC does not follow a single molecular pathway, but is rather characterized by multiple (overlapping) pathways [8].

In general, the genetic changes of sporadic colorectal carcinogenesis can be divided into chromosomal instability (CIN, 85% of cases) and microsatellite instability (MIN, 15% of cases). CIN-type tumors display many genomic alterations and are frequently aneuploid, whereas MIN-tumors have fewer genomic alterations and are generally regarded as diploid [17, 18]. Although DNA analysis by mCGH and FISH [6, 11–16] and mRNA expression profiling by cDNA microarray [19–23] have revealed CIN- and MIN-type tumor-associated candidate genes, there is currently little experimental evidence about the genome-wide differences of DNA copy number changes, especially at a gene-specific level, between CIN- and MIN-type colorectal cancers. Moreover, whether such gene-specific profiles of DNA copy number changes could be responsible for the differential mRNA expression profiles and the distinct clinicopathological phenotypes of CIN- and MIN-type colorectal tumors remains to be solved.

Recent studies have addressed the issue of screening DNA copy number changes by array-based comparative genomic hybridization (aCGH) in colorectal cancer [24–26]. These studies used fresh frozen tissues without prior microdissection of tumor cells, potentially biasing the quantity and quality of aCGH data. Moreover, no clear distinction was made between sporadic and potentially familiar colorectal cancer cases. With respect to differences of CIN- and MIN-type colorectal cancers, only one study [26], which included 125 cases, revealed distinct chromosomal regions affected in CIN- and MIN-type tumors. Similar chromosomal regions were detected in the other aCGH studies [24, 25], but sample sizes of CIN- and MIN-tumors were unbalanced and low (32 vs 2 and 4 vs 6, respectively) and didn't allow final conclusions about DNA copy number differences between CIN- and MIN-type tumors. Thus, candidate chromosomal regions were detected in CIN- and MIN-type colorectal cancers, but gene-specific targets, especially with respect to differences between CIN- and MIN-type colorectal cancers, await identification and validation.

To evaluate whether distinct genomic DNA alterations occur in CIN- compared to MIN-type sCRC and to pinpoint involved oncogenes and tumor suppressor genes, we therefore analyzed DNA from microdissected tumor cells of a previously characterized group of sporadic CIN- and MIN-type colorectal cancers [27] by aCGH. The aCGH platform included a selected panel of 287 target sequences, mostly known oncogenes and tumor suppressor genes, and allowed both reliable aCGH screening and subsequent validation of candidate genes by FISH using serial sections of formalin-fixed and paraffin-embedded tissues, as shown before for Barrett carcinomas [28]. aCGH revealed distinct DNA copy number changes between sporadic CIN- and MIN-associated colorectal carcinomas. A differential role of these candidate oncogenes and tumor suppressor genes in tumor development and progression of sporadic CIN and

MIN CRC is likely and may also be involved in the response or resistance to therapeutic interventions, such as that shown for microsatellite instability and 5-fluorouracil (5-FU) [29].

Materials and methods

Tissue samples

The study included a total of 22 cases with sCRC, of which the CIN and MIN status had been determined in a previous study [27]. Resection specimens were from the colon (19/22 cases) and from the rectum (3/22 cases). None of the patients had received neoadjuvant therapy before resection of the primary tumor and 21/22 cases had an R0 resection and 1/22 an R1 resection (rectum carcinoma and palliative surgery). Hematoxylin & eosin (HE) sections of the specimens had been classified for pT and pN categories [30] and WHO tumor type and tumor grade [31]. The clinicopathological data of all cases is summarized in Table 1.

Representative tissue areas (normal epithelium and invasive carcinoma) were marked on HE sections for microdissection (see below). Case-matched normal colonic epithelial cell and invasive carcinoma cells from the same formalin-fixed and paraffin-embedded surgical resection specimens were used for preparation of genomic DNA, but with normal epithelium being derived from the resection margins at a distance of at least 10 cm from the primary tumor.

Table 1 Clinicopathological data of cases

Case ID	Age	Sex	pT	pN	Hist. type	G	Genetic instability [27]
1	71	Male	3	0	Tubular	2	CIN
2	86	Male	3	0	Tubular	2	CIN
3	88	Male	3	0	Tubular	2	CIN
4	67	Male	3	0	Mucinous	3	MIN
5	74	Male	3	0	Tubular	3	CIN
6	64	Female	2	0	Tubular	2	CIN
7	70	Male	3	0	Tubular	2	CIN
8	83	Female	3	2	Tubular	3	MIN
9	90	Female	3	1	Tubular	2	MIN
10	77	Female	3	0	Tubular	2	CIN
11	75	Male	3	0	Tubular	2	CIN
12	80	Female	3	0	Tubular	2	MIN
13	90	Female	3	0	Mucinous	2	MIN
14	76	Male	3	0	Tubular	2	CIN
15	82	Male	3	0	Mucinous	3	MIN
16	66	Female	3	0	Undiff.	3	MIN
17	69	Female	3	0	Tubular	2	MIN
18	86	Female	2	0	Mucinous	2	MIN
19	72	Male	2	0	Undiff.	3	MIN
20	64	Male	3	1	Tubular	3	CIN
21	79	Female	3	0	Tubular	2	MIN
22	77	Male	3	0	Tubular	2	CIN

Microdissection and DNA isolation

Normal colorectal epithelium and invasive carcinoma cells were microdissected from each one 10 μm section under microscopic surveillance using fine needles. Cells were immediately placed into tissue lysis buffer (QIAamp DNA Kit, Qiagen, Hilden, Germany) and incubated overnight at 55°C. DNA was purified the next day according to the manufacture's protocol, eluted in 20 μl of water, and measured in a spectrophotometer (ND1000, Peqlab, Erlangen, Germany). In addition, DNA fragment length was assessed by agarose gel electrophoresis and showed good quality for aCGH analysis (Fig. 2a).

aCGH experiments and statistical data analysis

For aCGH, commercially available arrays, including 287 target clones of oncogenes and tumor suppressor genes spotted in triplicate ("GenoSensor™ Array 300," Abbott, Wiesbaden, Germany), were used according to the manufacturer's protocols with some modifications:

A pool of all 22 normal DNAs was used as "reference DNA" for the aCGH experiments. This approach had been successful before in our laboratory [28], but was additionally assessed in the present study by aCGH analysis of individual normal DNA against the pooled normal reference DNA. From this, the threshold for significant DNA copy number changes above normal variation was defined for DNA losses and gains at <0.8 and >1.2, respectively ("Results," Fig. 1).

For each aCGH experiment, 300 ng of pooled reference DNA and 300 ng of one tumor DNA were subjected to random priming with Cy3- and Cy5-labeled deoxycytidine 5′-triphosphates, respectively ("Microarray Random Priming Kit," Abbott). This was followed by a DNase digestion step, probe purification using microspin columns (S-200 HR, Amersham), and checking the reaction on an agarose gel, with all processed samples having an acceptable size range of 50–200 bp (Fig. 2b). For sample, hybridization, Cy3-labeled pooled reference DNA, and Cy5-labeled tumor DNA were incubated together at equal amounts with hybridization buffer containing Cot-1 DNA, denatured, and incubated on the aCGH at 37°C for 72 h. Microarrays were washed three times in 2×SSC/50% formamide at 40°C for 10 min each, three times in 1×SSC at room temperature for 5 min each, and rinsed in distilled water before embedding in diamidino-2-phenylindole (DAPI)-mounting medium. Microarrays were left for 45 min before

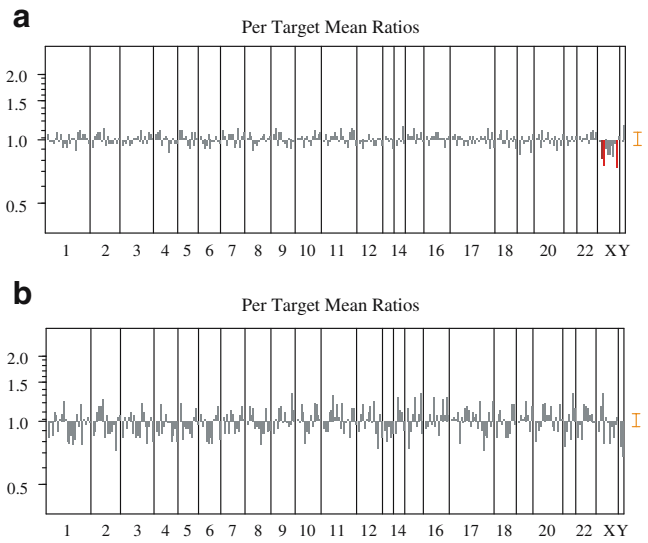

Fig. 1 Validation of aCGH approach. Control experiments for aCGH were performed as described in "Materials and methods" and "Results." aCGH data is depicted for all investigated target sequences (*bars* along *x*-axis) as the mean ratio (*y*-axis). **a** aCGH analysis of DNA extracted from microdissected normal colonic epithelium of a female case against normal colonic epithelial DNA of a male case. **b** aCGH analysis of normal colonic epithelial DNA from a single case vs the pooled normal reference DNA (normal epithelial DNA of all 22 cases). The colors used for each target sequence specific bar are *gray* (no significant DNA copy number change) and *red* (significant DNA copy number loss)

scanning in the "GenoSensor Reader System" (Abbott, Wiesbaden, Germany). DNA copy number changes from scanned microarrays were identified by the software and analysis program supplied (Abbott, Wiesbaden, Germany). This first segments and identifies target spots on the captured image and rejects debris, then measures the intensity and ratios of tumor to reference DNA signals hybridized to triplicate target clones, performs normalization, and finally statistically evaluates significant copy number changes. The thresholds for significant DNA copy number changes were for DNA losses of <0.8 and for DNA gains of >1.2. Differences of copy number changes between CIN and MIN tumors were evaluated by calculating the frequencies of aberration per target gene.

Validation of aCGH results by FISH analysis

To validate individual genes detected by aCGH, serial sections (5 μm) of a tissue microarray containing representative invasive carcinoma areas of all 22 sCRC cases were hybridized with gene-specific and centromere-specific (chromosome enumeration probe, CEP) fluorescent hybridization probes. A dual color probe for EGFR/CEP7 (Spectrum Orange™, Spectrum Green™) and a single color probe for ZNF217 (Spectrum Orange™) were commercially available (Abbott/Vysis, Wiesbaden, Germany). A STK15-specific single color probe (Cy3™, red) and a single color CEP20-specific probe [fluorescein isothiocyanate (FITC), green] were custom made (Chrombios, Raubling, Germany). The single color probes for ZNF217 and STK15 were each cohybridized with the single color CEP20 probe, so as to allow assessment of gene-specific (red) and centromere-specific (green) signals in the same cells.

Before probe hybridization, tissue sections were subjected to deparaffination (2× xylene and 100, 95, 70, and 50% ethanol) and pretreatment in citrate buffer (pH 6.0) in a microwave oven (180 W) for 20 min followed by Pronase E (0.05%) digestion for 3 min at 37°C. Subsequently, tissue sections were denatured in 50% formamide at room temperature for 15 min and in 70% formamide at 75°C for 5 min. Slides were immediately immersed in ice cold ethanol (70, 95, and 100%, 5 min each) and dried at 37°C. In the meantime, fluorescent hybridization probes had been denatured at 75°C for 5 min and were pipetted onto dried slides, followed by incubation for 16 h at 37°C. Slides were then washed in 2×SSC at room temperature and in 2×SSC at 37°C for each 2 min and finally counterstained with DAPI for 3 min. Cover-glassed (Vecta-Shield, Molecular Probes) slides were stored at −20°C until analysis.

Microscopic analysis of FISH sections was performed using a fluorescence microscope with ApoTome imaging system for 3D visualization ("Zeiss Axioplan2 imaging microscope" equipped with a PlanApochromat ×63/NA1.4 oil objective, Carl Zeiss MicroImaging GmbH, Göttingen, Germany). A total of 5–10 image stacks were taken with a pixel size of 1,388×1,040 at 0.925- to 0.945-μm intervals from representative tumor areas. The AxioVision software converted the image stacks into a 3D view, which was then assessed for the number of gene and centromere signals. Evaluation of FISH was done by an investigator who did not know the results of the aCGH. Signals of gene- and CEP-specific probes were counted in at least 50 cells and the mean ratio of gene/CEP signals was calculated for each case.

Results

Validation of aCGH approach

To validate sample preparation and aCGH analysis of the formalin-fixed and paraffin-embedded tissue samples, normal colonic epithelium DNA samples from a female were hybridized against those from a male case (Fig. 1a). No copy number changes were detected, except for losses at the X chromosome. The mean copy number change for the 287 targets was 1.0034±0.06 (coefficient of variation=2.53±2.06%). In addition, aCGH analysis of a single normal DNA against the pooled normal reference

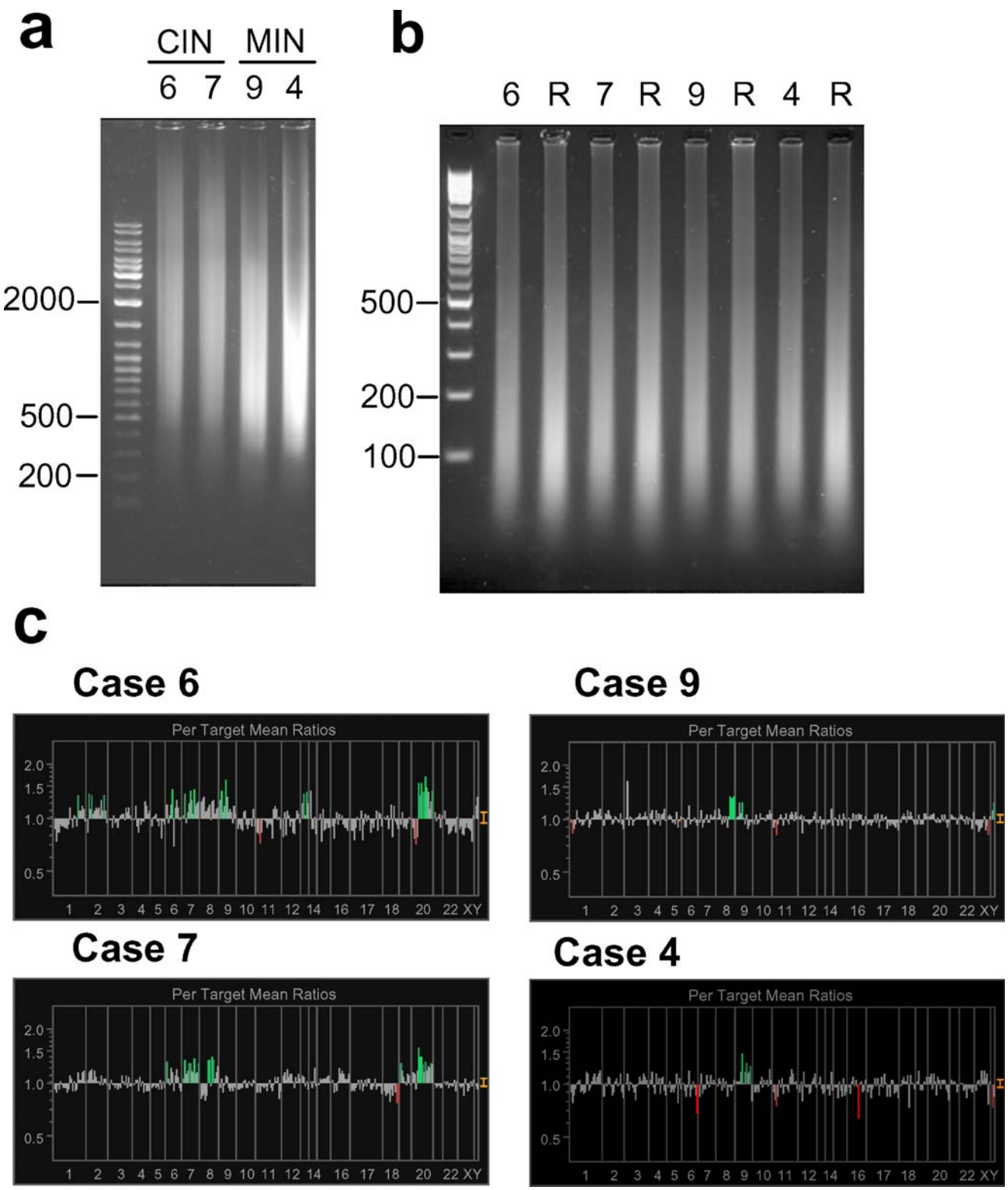

Fig. 2 Experimental steps for aCGH analysis of the sporadic colorectal carcinomas. Representative data of the experimental steps of aCGH analysis is shown for two CIN-type (*cases 6* and *7*) and two MIN-type (*cases 9* and *4*) tumors ("Materials and methods", Table 1): agarose gels of **a** purified DNA extracts from microdissected invasive tumor cells and **b** of labeled and fragmented tumor DNAs and pooled normal reference DNA (*R*), with fragment sizes indicated on the basepair ladders (*left lanes*). **c** aCGH profiles of the two CIN-type (*6* and *7*; *left panels*) and two MIN-type (*9* and *4*; *right panels*) tumor DNAs. aCGH data is depicted at all investigated target sequences (*bars* along *x*-axis) as the mean ratio (*y*-axis). The colors used for each target sequence specific bar are *gray* (no significant DNA copy number change), *green* (significant DNA gain), and *red* (significant DNA loss)

DNA revealed no significant copy number changes (Fig. 1b). However, a slightly higher variation of signals (mean copy number change for 287 targets=1.0156±0.17, coefficient of variation=2.02±1.45%) was detected and the threshold for significant DNA losses and gains was adapted accordingly to <0.8 and >1.2, respectively.

Evaluation of aCGH profiles of sCRC

All 22 cases with sCRC (Table 1) were analyzed as described in the "Materials and methods" and the individual steps of the analysis are exemplified for each two CIN- and MIN-type tumors in Fig. 2.

Purified DNA from microdissected, formalin-fixed, and paraffin-embedded invasive colorectal tumor cells did exhibit an acceptable fragment size of >200 bp (Fig. 2a). After fluorescence labeling and DNase digestion, both tumor DNAs and simultaneously processed reference DNAs had comparable fragment sizes between 50 and 200 bp (Fig. 2b), and these resulted in high quality aCGH data (Fig. 2c, coefficient of variation=2.05±2.7% for all 22 tumors).

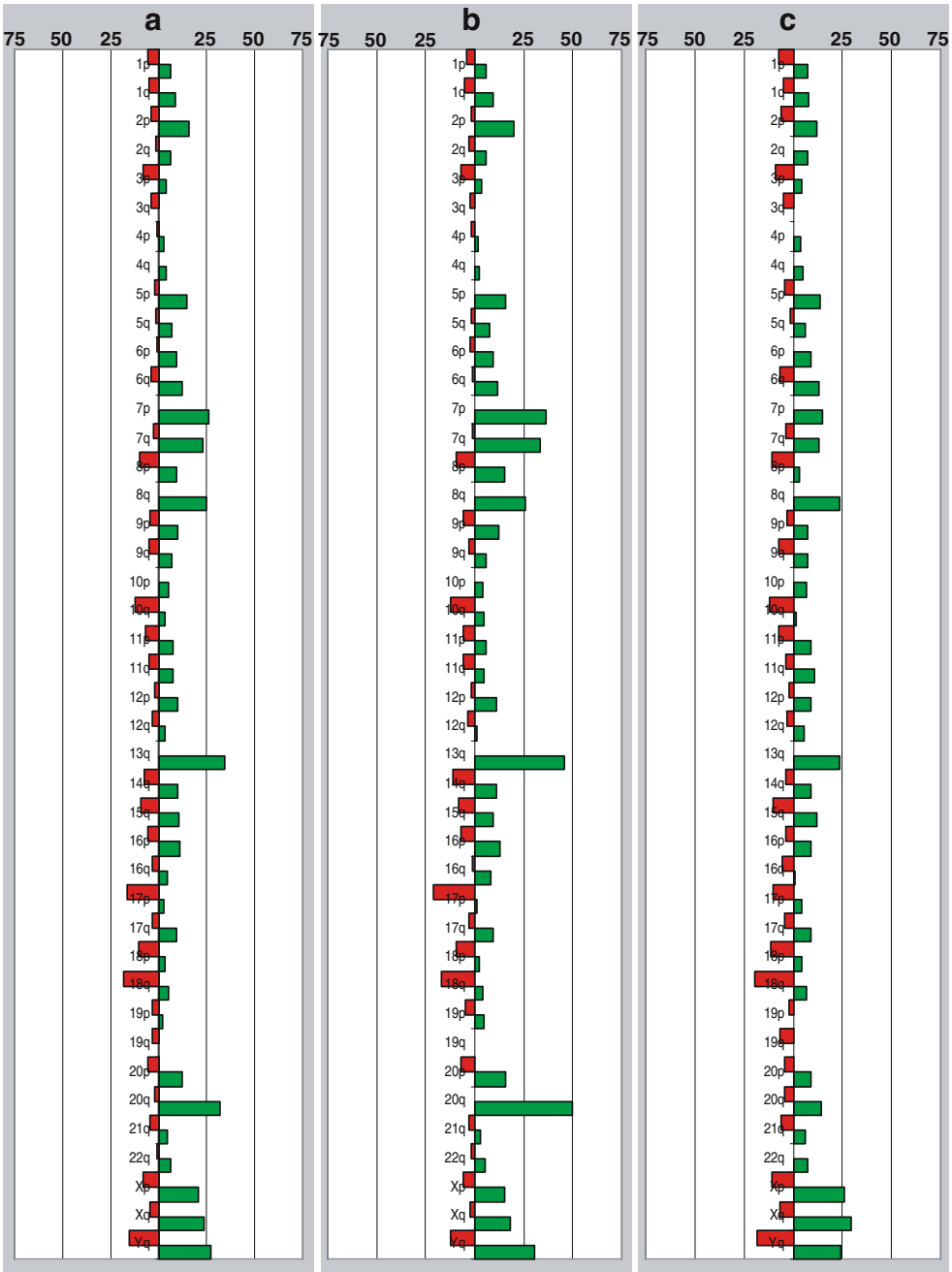

Fig. 3 Summary of DNA copy number changes detected in 22 sporadic colorectal carcinomas by aCGH. The graphs display the distribution of DNA copy number changes of the investigated target sequences, summarized into the p and q arms of chromosomes 1–22 and chromosomes X, Y (*y*-axis). **a** Frequency of DNA copy number changes of all 22 sporadic colorectal carcinomas combined and frequency of DNA copy number changes in the separate groups of **b** only CIN-type tumors and **c** only MIN-type tumors. The mean frequency is expressed as percentage (*x*-axis) and shown as *red bars* for DNA losses (*left bars*) and as *green bars* for DNA gains (*right bars*)

Analysis of all DNA copy number changes from the 22 tumor DNAs showed that chromosomal regions known to be affected in colorectal cancers (metaphase CGH [6, 11–16]) were also identified in the present aCGH approach. Thus, aCGH revealed frequent DNA gains at 20q, 13q, 8q, and 7p and losses at 18q, 17p, and 8p (Fig. 3a) and specifically pinpointed oncogenes and tumor suppressor genes located within these chromosomal regions (Tables 2 and 3).

Table 2 Gene-specific DNA amplifications detected in sporadic colorectal carcinomas by aCGH

Gene name	Location	CRC	CIN	MIN
U32389	2p tel	36.36	36.36	36.36
D5S23	5p15.2	31.82	27.27	36.36
G31341	7p tel	36.36	45.45	27.27
IL6	7p21	36.36	54.55	18.18
RFC2, CYLN2	7q11.23	36.36	63.64	9.09
MET	7q31	22.73	36.36	9.09
7QTEL20	7q tel	40.91	72.73	9.09
D8S596	8p tel	22.73	36.36	9.09
E2F5	8p22–q21.3	36.36	27.27	45.45
EXT1	8q24.11–q24.13	31.82	45.45	18.18
MYC	8q24.12–q24.13	36.36	45.45	27.27
AFM137XA11	9p11.2	40.91	36.36	45.45
INS	11p tel	31.82	27.27	36.36
GARP	11q13.5–q14	31.82	18.18	45.45
ATM	11q22.3	27.27	18.18	36.36
BRCA2	13q12–q13	36.36	54.55	18.18
RB1	13q14	40.91	45.45	36.36
D13S319	13q14.2	36.36	45.45	27.27
D13S25	13q14.3	36.36	54.55	18.18
WI-5214	15q tel	31.82	27.27	36.36
16PTEL03	16p tel	31.82	27.27	36.36
DCC	18q21.3	31.82	27.27	36.36
20PTEL18	20p tel	27.27	36.36	18.18
TOP1	20q12–q13.1	31.82	54.55	9.09
NCOA3 (AIB1)	20q12	31.82	54.55	9.09
MYBL2	20q13.1	31.82	54.55	9.09
CSE1L (CAS)	20q13	27.27	45.45	9.09
PTPN1	20q13.1–q13.2	22.73	36.36	9.09
STK6 (STK15)	20q13.2–q13.3	36.36	54.55	18.18
ZNF217 (ZABC1)	20q13.2	31.82	45.45	18.18
CYP24	20q13.2	36.36	54.55	18.18
TNFRSF6B (DCR3)	20q13	31.82	36.36	27.27
TPD52L2, TOM	20q tel	27.27	45.45	9.09
20QTEL14	20q tel	40.91	63.64	18.18
KAL	Xp22.3	36.36	27.27	45.45
XIST	Xq13.2	36.36	27.27	45.45
SRY	Yp11.3	36.36	36.36	36.36
AZFa region	Yq11	31.82	36.36	27.27

The frequency of DNA amplification occurring at a specific target sequence is shown in percent (n/N cases×100). Only alterations occurring in at least 30% of the cases are listed.

CRC All 22 sCRC cases, *CIN* CIN-type tumors (n=11), *MIN* MIN-type tumors (n=11)

Comparison of aCGH profiles between CIN- and MIN-type colorectal cancers

Upon separate evaluation of the CIN- and MIN-type tumor aCGH profiles, differences were observed between the two groups for the frequency of DNA gains and losses at specific chromosomal regions and for gene-specific amplifications and deletions (Fig. 3b,c, Tables 2 and 3).

With respect to the frequency of specific chromosomal regions, those previously associated with colorectal tumors by mCGH [6, 11–16], i.e., DNA gains at 20q, 13q, 8q, and 7p and losses at 18q, 17p, and 8p, were more often observed in CIN-type (Fig. 3b), but less in MIN-type (Fig. 3c) tumors. In particular, DNA gains at 20q were detected at a frequency of 36–64% in CIN-type, but only 9–27% in MIN-type tumors; DNA gains at 13q were identified at a frequency of 45–54% in CIN- and only 18–36% in MIN-type tumors; DNA amplifications at 7pq were seen at a frequency of 9–72% in CIN- and only 9–27% in MIN-type tumors and DNA losses at 17p occurred at 18–55% in CIN- and only 9–18% in MIN-type tumors.

Moreover, aCGH allowed the identification of specific target sequences within these differentially altered chromosomal regions. Thus, genes preferentially altered within the

Table 3 Gene-specific DNA deletions detected in sporadic colorectal carcinomas by aCGH

Name	Location	CRC	CIN	MIN
3PTEL25	3p tel	13.64	0.00	27.27
RAF1	3p25	13.64	0.00	27.27
THRB	3p24.3	31.82	36.36	27.27
PDGRL	8p22–p21.3	22.73	18.18	27.27
LPL	8p22	22.73	27.27	18.18
EGR2	10q21.3	22.73	27.27	18.18
DMBT1	10q25.3–q26.1	22.73	27.27	18.18
WI-6509	11q tel	22.73	27.27	18.18
MAP2K5	15q23	31.82	45.45	18.18
282M15/SP6	17p tel	22.73	27.27	18.18
HIC1	17p13.3	31.82	45.45	18.18
LLGL1	17p12–17p11.2	36.36	54.55	18.18
FLI, TOP3A	17p12–17p11.2	22.73	27.27	18.18
SHGC17327	18p tel	27.27	27.27	27.27
LAMA3	18q11.2	13.64	27.27	0.00
DCC	18q21.3	18.18	9.09	27.27
BCL2 3′	18q21.3	22.73	18.18	27.27
CTDP1, SHGC-145820	18q tel	45.45	45.45	45.45
PCNT2 (KEN)	21q tel	18.18	9.09	27.27
STS 3′	Xp22.3	22.73	18.18	27.27
SRY	Yp11.3	27.27	18.18	36.36

The frequency of DNA deletion occurring at a specific target sequence is shown in percent (n/N cases×100). Only alterations occurring in at least 20% of the cases are listed.

CRC All 22 sCRC, *CIN* CIN-type tumors (n=11), *MIN* MIN-type tumors (n=11)

identified CIN-tumor-associated chromosomal regions (20q, 13q, 7pq, and 17p) included DNA amplifications of eight genes on chromosome 20q (TOP1, AIB1, MYBL2, CAS, PTPN1, STK15, ZNF217, and CYP24), two genes on chromosome 13q (BRCA2 and D13S25), and three genes on chromosome 7 (IL6, CYLN2, and MET) as well as DNA deletions of two genes on chromosome 17p (HIC1 and LLGL1) (Tables 2 and 3).

Finally, additional differences of gene-specific DNA amplifications and deletions between CIN- and MIN-type tumors were observed at other chromosomal regions than 20q, 13q, 7pq, and 17p (Tables 2 and 3): CIN-tumor-associated DNA amplifications were identified for EXT1 (8q24.11) and MYC (8q24.12) as well as DNA deletions for MAP2K5 (15q23) and LAMA3 (18q11.2). In contrast, distinct MIN-tumor-associated DNA amplifications were detected for E2F5 (8p22–q21.3), GARP (11q13.5–q14), ATM (11q22.3), KAL (Xp22.3), XIST (Xq13.2), and DNA deletions for RAF1 (3p25), DCC (18q21.3), and KEN (21q tel).

Validation of aCGH findings by FISH

To validate aCGH findings, FISH analysis of three selected genes was performed on serial tissues as those used for aCGH, including one gene showing normal DNA copy numbers in CIN- and MIN-type tumors (EGFR on chromosome 7p) and two genes (STK15 and ZNF217) on chromosome 20q, which were differentially altered in CIN- and MIN-type tumors. Representative FISH images are shown with corresponding aCGH data and FISH ratios for two cases (#14=CIN and #16=MIN, see also Table 1) for each of the three genes in Fig. 4. Normal DNA copy numbers of EGFR as determined by aCGH in both CIN- and MIN-type tumors corresponded to normal EGFR copy numbers or polyploidy of chromosome 7 by FISH (Fig. 4a,d). Preferential amplification of STK15 (Fig. 4b,e) and ZNF217 (Fig. 4c,f) in CIN-type tumors as determined by aCGH was confirmed by FISH analysis. Note that aCGH data and FISH ratios are different parameters, as aCGH is based on a comparative analysis of DNA extracted from normal colorectal epithelium and microdissected tumor cells, whereas FISH is a direct cell-specific analysis and quantification is different for the two methods ("Materials and methods").

Discussion

To our knowledge, the present study is the first to have analyzed DNA copy number changes of a large series of mostly known oncogenes and tumor suppressor genes in CIN and MIN unstable sporadic colorectal carcinomas.

Several other studies have investigated CIN- and MIN-type colorectal cancers with respect to their mRNA expression profiles using cDNA microarrays [19–23] or to genome-wide DNA copy number changes using aCGH [24–26] or the relationship between aCGH and cDNA profiles [32]. Besides contributing to a better understanding of colorectal carcinogenesis and progression, such studies may become of relevance for dissection of the differential clinical responses of CIN- and MIN-type colorectal tumors to particular chemotherapeutic agents, such as 5-FU [29]. One major pitfall of these studies is the use of inhomogeneous patient groups (e.g., sporadic and familiar cases) and DNA extracted from tissue samples without prior microdissection. However, the latter is especially important for the detection of tumor-cell-specific DNA alterations because cancer resection specimens contain a highly variable number of tumor cells and associated stromal components.

Our study aim was therefore to provide a genome-wide profile of tumor-cell-specific DNA copy number changes in sCRC and to define CIN- and MIN-type-specific oncogenes and tumor suppressor genes, which most likely contribute to the deregulation of functional pathways as those seen on the mRNA level by cDNA microarrays. To achieve this, we included an equal number of previously well characterized CIN- and MIN-type sCRC [27]. Second, we used an aCGH approach with a small number (287) of target sequences [28] to avoid a potential bias introduced by large-scale screening of DNA alterations in small sample series. Besides telomeric sequences and microsatellite markers, these target sequences included mostly known oncogenes and tumor suppressor genes, which readily allowed candidate-specific validation. Third, aCGH was applied to microdissected matched normal colorectal epithelium and invasive tumor cells from 22 surgically resected, formalin-fixed, and paraffin-embedded tissue specimens. Fourth, we used reference DNA extracted from normal colorectal epithelium of the same resection specimens and hence tissues that had been equally processed as microdissected tumor cells. This avoided potential false positive or false negative results due to differential quality of test and reference DNAs. Finally, FISH validation of three genes was performed on serial tissues as those used for aCGH.

By doing this, aCGH profiles obtained from the 22 sporadic colorectal carcinomas, irrespective of their CIN or MIN status, confirmed DNA gains at 20q, 13q, 8q, and 7p and losses at 18q, 17p, and 8p chromosomal regions also previously reported by metaphase CGH [6, 11–16] and recent aCGH studies [24–26]. However, chromosomal regions 20q, 13q, 7, and 17p were preferentially altered in CIN-type tumors. Novel candidate oncogenes and tumor suppressor genes located within these CIN-type tumor-associated chromosomal regions were identified and included the amplified genes TOP1, AIB1, MYBL2, CAS,

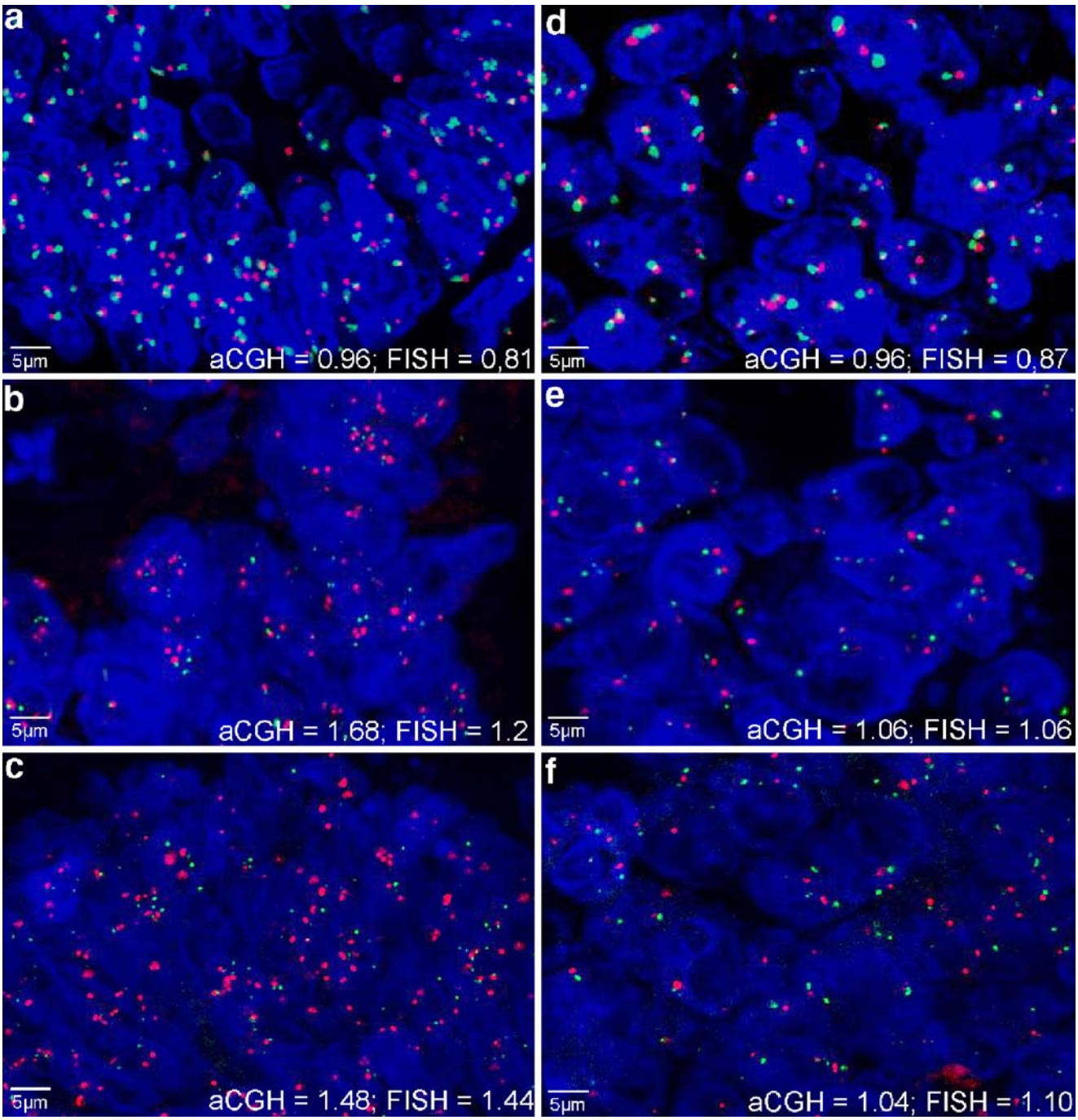

Fig. 4 Validation of aCGH data by FISH analysis. FISH images of two representative cases, one CIN-type (*case 14*, **a**–**c**) and one MIN-type (*case 16*, **d**–**f**) tumor are shown for three genes. FISH was performed as in "Materials and methods," with gene-specific probes detected as *red* signals and centromere-specific probes as *green* signals. Both aCGH data and FISH ratios (gene- to centromere-specific signals, "Materials and methods") are given for each case. Note that aCGH and FISH values cannot be directly compared ("Results"). Note also that there is a close structural association between gene- and centromere-specific signals of FISH analysis for cases without DNA copy number changes in aCGH (**a**, **d**, **e**, **f**), but a slight dislocation of gene- and centromere-specific signals for cases with DNA amplification (**b**, **c**). Subpanels **a** and **d** demonstrate that "normal" copy numbers of EGFR on chromosome 7p12, as determined by aCGH, were due to polyploidy in both CIN-type (**a**) and MIN-type (**d**) tumors. Subpanels **b**, **c**, **e,** and **f** show that the preferential STK15 and ZNF217 gene amplification on chromosome 20q in CIN-type tumors as seen by aCGH was confirmed by STK15-specific (**b**) and ZNF217-specific (**c**) FISH analysis in CIN-type, but not MIN-type tumors [STK15 (**e**) and ZNF217 (**f**)]

PTPN1, STK15, ZNF217, and CYP24 on chromosome 20q, BRCA2 and D13S25 on chromosome 13q and IL6, CYLN2, and MET on chromosome 7 as well as the deleted genes HIC1 and LLGL1 on chromosome 17p. Furthermore, additional CIN-tumor-associated gene amplifications were identified for EXT1 (8q24.11) and MYC (8q24.12) and gene deletions for MAP2K5 (15q23) and LAMA3 (18q11.2). In contrast, distinct MIN-tumor-associated DNA amplifications were detected for E2F5 (8p22–q21.3), GARP (11q13.5–q14), ATM (11q22.3), KAL (Xp22.3), and XIST (Xq13.2) and DNA deletions for RAF1 (3p25), DCC (18q21.3), and KEN (21q tel).

The preferential amplification of 8/11 investigated target sequences on 20q in CIN tumors is of particular interest, especially as the relevance of this amplicon in colorectal cancer is supported by other studies of early dysplastic lesions [6, 15] and colorectal liver metastasis [6, 14]. Of these eight amplified candidate genes, three (STK15, ZNF217, and CYP24) had also been detected in colorectal carcinomas in an independent aCGH study [26], but without showing differences between CIN- and MIN-type tumors. Whereas little is known about the functional role of ZNF217 and CYP24 in colorectal cancer, STK15 (20q13.2, Aurora-A) is a known oncogene [33] and overexpression of this centrosome-associated kinase was detected in genetic instable solid tumors [34, 35], including colorectal cancer [36, 37]. Moreover, our own studies [27] showed higher STK15-specific mRNA expression in CIN- than MIN-type colorectal tumors. Together, it is therefore likely that the preferential overexpression of STK15 mRNA in sporadic CIN-type tumors is the result of STK15-specific DNA amplification. Further investigations of the mechanisms of STK15 gene alterations and associated STK15 mRNA and protein expression in CIN- and MIN-type colorectal tumors and dysplastic precursor lesions will also be of high clinical relevance in view of the novel therapeutic strategies of Aurora kinase inhibition [38, 39].

Another highly relevant finding of the present aCGH study relates to the 17p chromosomal region. The involvement of this chromosomal region in colorectal cancer, in particular the p53 gene (17p13.1), was extensively studied. The present study has now shown the preferential loss of this region in CIN-type colorectal tumors and has pinpointed two novel candidate genes, HIC1 (17p13.3) and LLGL1 (17p12), preferentially deleted in CIN-type tumors. It is interesting to note that HIC1 encodes for a transcription factor that can interact with p53-induced cellular functions and mice with a heterogeneous disruption of HIC1 that spontaneously develop epithelial tumors (male) and soft tissue tumors and lymphomas (female) [40, 41]. In contrast, LLGL1 encodes for a structural protein, which contributes to cellular polarity and which was shown to interact with the WNT pathway in model system [42, 43]. In fact, Schimanski et al. [44] recently showed that downregulation of LLGL1 appears to contribute to progression of human colorectal cancer. However, the detailed functional effect of the differential DNA copy number changes of HIC1 and LLGL1 in CIN- and MIN-type colorectal tumors and their potential cross-talk to the p53 and WNT pathways remain to be resolved.

In conclusion, aCGH revealed distinct DNA copy number changes between sporadic CIN- and MIN-associated colorectal carcinomas. A differential role of these candidate oncogenes and tumor suppressor genes in tumor development and progression of sporadic CIN and MIN CRC is likely and may also be involved in the response or resistance to therapeutic interventions, such as shown for microsatellite instability and 5-FU [29].

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