

Chromosomal and methylation alterations in sporadic and familial adenomatous polyposis-related duodenal carcinomas

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Primary carcinomas of the small intestine are rare and the mechanism of their pathogenesis is poorly understood. Patients with familial adenomatous polyposis (FAP) have a high risk of developing duodenal carcinomas. The aim of this study is to gain more insight into the development of duodenal carcinomas. Therefore, five FAP-related duodenal carcinomas were characterized for chromosomal and methylation alterations, which were compared to those observed in sporadic duodenal carcinomas. Comparative genomic hybridization (CGH) and methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) was performed in 10 primary sporadic and five primary FAP-related duodenal carcinomas. In the FAP-related carcinomas, frequent gains were observed on chromosomes 8, 17 and 19, whereas in sporadic carcinomas they occurred on chromosomes 8, 12, 13 and 20. In 60% of the sporadic carcinomas, gains in the regions of chromosome 12 were observed which were absent in the FAP-related carcinomas ($P=0.04$). Hypermethylation was observed in the *immunoglobulin superfamily genes member 4 (IGSF4)*, *TIMP metalloproteinase inhibitor 3 (TIMP3)*, *Estrogen receptor 1 (ESR1)*, *adenomatous polyposis coli (APC)*, *H-cadherin (CDH13)* and *paired box gene 6 (PAX6)* genes. Hypermethylation of *PAX6* was only observed in FAP-related carcinomas (3/5) and not in sporadic carcinomas ($P=0.02$). In conclusion, in contrast to sporadic duodenal carcinomas, gains on chromosome 12 were not observed in duodenal carcinomas of patients with FAP. Identification of the genes in these regions of chromosome 12 could lead to a better understanding of the carcinogenesis pathways leading to sporadic and FAP-related duodenal carcinomas. Furthermore, hypermethylation seems to be a general feature of both FAP-related duodenal carcinomas as well as sporadic duodenal carcinomas with the exception of the *PAX6* gene, which is methylated only in FAP-related carcinomas.

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Although the small intestine is located between the stomach and the colon, both regions with a high cancer risk, carcinomas of the small bowel are surprisingly rare. In the Netherlands, the average annual incidence rate of small bowel carcinomas is approximately one case per 100 000 inhabitants, compared to 49 for colorectal cancer and 14 for gastric cancer.¹ In contrast to colorectal cancer, the molecular pathogenesis of small bowel tumors is

rarely the subject of research. In the small bowel, the most frequent site for the development of adenocarcinomas is the duodenum ($\pm 50\%$), followed by the jejunum ($\pm 25\%$) and the ileum ($\pm 13\%$) (rest is not specified).^{2–4} Patients with familial adenomatous polyposis (FAP),⁵ Crohn's disease,⁶ celiac disease⁷ or hereditary nonpolyposis colorectal cancer (HNPCC)⁸ are known to have a higher risk of developing small bowel carcinomas.

In patients with FAP, the duodenum is the main site for malignant transformation of mucosal cells in the small intestine. FAP is an autosomal dominant disorder caused by germline mutations in the tumor suppressor gene *adenomatous polyposis coli (APC)*.⁹ Patients initially develop hundreds to thousands of colorectal adenomas. Without prophylactic colect-

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omy, FAP will inevitably lead to colorectal cancer at a relatively young age.¹⁰ Nowadays, with increased survival due to prophylactic colectomy, problems of extra-colonic manifestations in these patients become apparent.¹¹ At present, the prevalence of duodenal carcinoma in patients with FAP is 2–5%.^{11–14} Compared to the general population, the relative risk of duodenal adenocarcinoma is exceptionally high (relative risk, 331; 95% confidence limits: 133–681).¹⁵ Together with desmoid tumors, duodenal carcinomas are now the leading causes of cancer-related mortality in patients with FAP.¹⁶

Deoxyribonucleic acid (DNA) changes are crucial steps in tumor initiation and progression.¹⁷ Next to mutations in oncogenes and tumor suppressor genes, alterations in DNA copy numbers and DNA methylation patterns have been observed as common changes in colorectal and gastric cancer. Copy number changes can lead to increased or decreased gene expression whereas mutations can have an activating or inactivating effect. Besides these genetic changes, epigenetic changes, such as DNA methylation, may result in altered gene-expression levels. Usually, aberrant methylation of normally unmethylated CpG-rich areas, also known as CpG islands, which are located in the promoter regions of genes, have been associated with transcriptional inactivation of important tumor suppressor genes, DNA repair genes or metastasis inhibitor genes.^{18,19}

Until now, only a few reports have been published on chromosomal changes or methylation of DNA with respect to the carcinogenesis of the small intestine,^{20–23} which lead us to use global screening methods in this study. Comparative genomic hybridization (CGH) enables detection of chromosomal gains and losses, and can be applied to fixed tissue samples. Major advantages of CGH are that it does not require preknowledge about the genetic constitution of the tumor tissue and the entire genome is analyzed in one single experiment.²⁴ Multiplex ligation-dependent probe amplification (MLPA) has been accepted as a simple and reliable method for detection of copy number changes in paraffin-embedded tumor tissue.²⁵ Recently, this technique was adjusted allowing methylation-specific analysis (methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)).²⁶ A major advantage over the conventionally used techniques analyzing the methylation status is that multiple loci can be analyzed simultaneously using formalin-fixed and paraffin-embedded tissue.

So far, no comparison has been made between chromosomal and methylation alterations in sporadic vs FAP-related duodenal tumors. The aim of this study is to gain more insight into the development of duodenal carcinomas. FAP-related duodenal carcinomas were therefore characterized for chromosomal and methylation alterations, which were compared to those observed in sporadic duodenal carcinomas.

Materials and methods

Patients

In the period 1991–2004, five primary duodenal carcinomas of patients with FAP (average age 54 ± 9 years) and 10 primary sporadic duodenal carcinomas (average age 63 ± 19 years) were retrieved from the files of the Departments of Pathology (Radboud University Nijmegen Medical Centre, Nijmegen and Rijnstate Hospital, Alysis, Arnhem, the Netherlands). Non-neoplastic duodenal tissue was included to serve as control DNA in the CGH. The patients with FAP were diagnosed on their clinical characteristics: namely, the presence of hundreds of adenomas in their colon. The Local Medical Ethical Review Committee approved this study.

Histological Evaluation of the Carcinomas

Classification of tumors was performed using the World Health Organization (WHO) guidelines; a tumor was considered mucinous when over 50% of the adenocarcinoma was mucinous.²⁷ Histological differentiation was categorized into well, moderately, poorly, or undifferentiated adenocarcinomas based on the part of poorest differentiation in the tumor, excluding the invasive front.²⁸ Growth pattern and peritumoral inflammation was assessed according to Jass *et al.*²⁹ Fibroblastic reaction and intratumoral inflammation were scored as none, little, moderate, or extensive.

DNA Isolation

At least 10 sections (20 μ m) of macro-dissected (>80% tumor cells) formalin-fixed and paraffin-embedded tumor tissue were collected and incubated in 125 μ l P-buffer (50 mM Tris-HCl pH 8.2, 100 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 0.5 % (v/v) Tween 20, 0.5 % (v/v) NP40, 20 mM dithiothreitol (DTT) for 15 min at 90°C. Protein digestion was performed by adding proteinase K (Roche Diagnostics GBMH, Mannheim, Germany) with a final concentration of 0.5 mg/ml. The samples were incubated at 55°C for 24 h, followed by incubation at 37°C for 48 h with addition of 5 μ l fresh proteinase K (20 mg/ml) every 24 h. Subsequently, DNA was purified using the DNeasy tissue kit (Qiagen, Venlo, the Netherlands). The isolation was performed according to the instructions of the manufacturer with the modifications of adding 250 μ l ethanol in step 4 and repeating step 7 with buffer AW2. The DNA concentration was measured by using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Comparative Genomic Hybridization

All tumors were genetically characterized by conventional CGH detecting copy number changes

>2 Mb as described previously.^{30–32} In short, all DNA samples isolated from control- and tumor tissues were labeled by nick-translation with digoxigenin-deoxyuridine 5-triphosphate (dUTP) and biotin-dUTP, respectively (Roche Molecular Biochemicals, Almere, the Netherlands), and precipitated in the presence of 50 × human COT-1 DNA (Gibco BRL Life Technologies Inc., Gaithersburg, MD, USA) and herring sperm DNA (Invitrogen, Carlsbad, CA, USA). The probe and the metaphase slides were denatured simultaneously. After hybridization and post-hybridization washes, biotin and digoxigenin were detected using streptavidin-fluorescein isothiocyanate (FITC) and sheep-anti-digoxigenin-tetramethylrhodamine isothiocyanate (TRITC) (Roche Molecular Biochemicals). The chromosomes were counterstained with 4,6'-diamino-2-phenylindole-dihydrochloride (DAPI) (Merck, Darmstadt, Germany) and the slides were mounted in Fluoroguard (Biorad, Veenendaal, the Netherlands). For CGH analysis, Quips CGH software (Applied Imaging, Newcastle upon Tyne, UK) was used. Detection thresholds for losses and gains were set at 0.8 and 1.2, respectively. For clear copy number changes, the thresholds were 0.6 and 1.4 and a ratio larger than 1.6 indicated high copy number amplifications. The average of approximately 10 metaphases was used to calculate the ratio profiles of the chromosomes.

Methylation-Specific Multiplex Ligation-Dependent Probe Amplification

This technique uses multiple probe sets each consisting of two oligonucleotides, both containing a sequence-specific region used for hybridization to the genomic test DNA, tagged with common tails complementary to a universal primer set. One of both oligonucleotides additionally contains a stuffer sequence of a characteristic length, allowing separation of the individual loci (probe sets) analyzed. The probe mix, containing multiple probe sets is hybridized onto the genomic test DNA. In one part of the sample adjacently hybridized oligonucleotides are joint through ligation, whereas for the other half of the sample ligation is combined with a methylation-sensitive restriction enzyme *HhaI* (recognition site GCGC) digesting the unmethylated fragments, ligation and ligation-digestion sample, respectively. Ligated probe sets are amplified by polymerase chain reaction (PCR) and subjected to capillary electrophoresis. By comparison of the ligated sample (indicative for the amount of total DNA, methylated as well as unmethylated, with the ligation-digestion sample (indicative for the amount of methylated DNA), the amount of methylation can be calculated. For methylation analysis, probe mixes ME001 and ME002 were purchased from MRC-Holland (Amsterdam, the Netherlands). The probe mix contains 25 probe sequences of which 15

sequences (control probes) are not influenced by *HhaI* digestion. All MLPA probe pairs code for unique human single copy DNA sequences and were designed and prepared as described by Schouten *et al.*³³ Probe sequences, gene loci and chromosome locations can be found at www.mlpa.com. MLPA was performed as described by the manufacturer with minor modifications. In short, DNA (100–200 ng) was dissolved in 5 μl TE-buffer (10 mM Tris pH 8.2, 1 mM EDTA pH 8.0), denatured and subsequently cooled down to 25°C. After adding the probe mix, the sample was denatured and the probes were allowed to hybridize (16 h at 60°C). Subsequently, the samples were divided in two and one half of the samples was ligated, whereas for the other part of the samples ligation was combined with the *HhaI* digestion enzyme. This digestion resulted in ligation of only the methylated sequences. PCR was performed on both parts of the samples in a volume of 50 μl containing 10 μl of the ligation reaction mixture using the PTC 200 thermal cycler (MJ Research Inc., Waltham, MA, USA) 33 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 30 s and extension at 72°C for 1 min with a final extension of 20 min at 72°C. An additional agarose gel electrophoresis was used to check MLPA efficiency.³⁴ Aliquots of 1 μl of the PCR reaction were combined with 0.3 μl LIZ-labeled internal size standard (Applied Biosystems, Foster City, CA, USA) and 8.7 μl deionized formamide. After denaturation, fragments were separated and quantified by electrophoresis on an ABI 3730 capillary sequencer and Genemapper analysis (both Applied Biosystems). Peak identification was checked visually and values corresponding to peak size in base pairs (bp) and peak heights were used for further data processing. Instead of peak height, peak area can also be used.²⁵ The validity of the probes was checked by the analysis of normal DNA. Furthermore, the sensitivity of ME001 and ME002 was established in a titration experiment in which normal DNA isolated from lymphocytes, which then was methylated *in vitro* using SSSI (New England Biolabs, Ipswich, MA, USA) as described by the manufacturer. Methylated samples were diluted to 75% methylated (M), 50% M and 25% M using the original unmethylated DNA.³⁵ Data analysis was performed in Excel as described by the manufacturer of the MLPA kits. First, the fraction of each peak is calculated by dividing the peak value of each probe amplification product by the combined value of the control probes within the sample, this to compensate for differences in PCR efficiency of the individual samples. For hypermethylation analysis this 'relative peak value' or the so-called 'probe fraction' of the ligation-digestion sample is divided by the 'relative peak value' of the corresponding ligation sample, resulting in a so-called 'methylation-ratio' (M-ratio). Aberrant methylation was scored when the calculated M-ratio was >0.25. The ME001 kit included the following genes:

PTEN, CD44, GSTP1, ATM, IGSF4, CDKN1B, CHFR, BRCA2, CDH13, HIC1, BRCA1, TP73, TIMP3, CASP8, FHIT, MLH1 (2 probes, *MLH1a* and *MLH1b*), *RASSF* (2 probes, *RASSF1a* and *RASSF1b*), *RARB, VHL, APC, ESR1, CDKN2A, CDKN2B* and *DAPK1*. And the ME002 kit included the following genes: *PTEN, MGMT* (2 probes, *MGMT-a* and *MGMT-b*), *CD44, WT1, GSTP1, ATM-a, IGSF4-a, STK11, CHFR, BRCA2, RB1* (2 probes, *RB1-a* and *RB1-b*), *THBS1, ASC, CDH13, TP53, BRCA1, TP73, GATA5, RARB, VHL, ESR1, PAX5A, CDKN2A* and *PAX6*.

Statistical Analysis

The Wilcoxon–Mann–Whitney test was used to compare the age of the patients and changes in copy numbers or number of hypermethylated genes in tumors of patients with FAP vs those of patients with sporadic cancer. Fisher's exact test was used to examine differences between FAP-related and sporadic carcinomas in the frequencies of chromosomal copy number changes and percentage carcinomas showing methylation. The χ^2 test was used for the association between copy numbers or methylation and histopathological characteristics. $P < 0.05$ was considered to be statistically significant (SPSS 12.0.1 for Windows 2003, SPSS Inc., Chicago, IL, USA).

Results

Histological Evaluation of the FAP-Related Tumors

The histopathological characteristics of the five FAP-related and 10 sporadic carcinomas are summarized in Table 1. All FAP-related carcinomas were adenocarcinomas with moderate-to-poor differentiation. One carcinoma of the intestinal type

was located in the ampullary region. Three carcinomas showed a circumscriptive growth pattern and two carcinomas a diffuse growth pattern. There was none to little peritumoral inflammation in four FAP-related carcinomas. Moderate-to-extensive intratumoral inflammation was observed in two carcinomas and fibroblastic reaction was present in three carcinomas. In the majority of carcinomas invasion through the bowel wall was present (stage T3/T4). In three patients, lymph node metastases were present.

Histological Evaluation of the Sporadic Tumors

Three carcinomas were located in the ampullary region (*intestinal type*). One sporadic carcinoma was a mucinous carcinoma. The adenocarcinomas were poorly (5/10), moderately (2/10) and well (2/10) differentiated. Circumscribed and diffuse growing carcinomas were observed in five and four of the patients, respectively. Peritumoral as well as intratumoral inflammation was moderate-to-extensive in four tumors. A fibroblastic reaction was present in nine carcinomas at a moderate-to-extensive rate. Most sporadic carcinomas (9/10) were in an advanced stage (T3/T4) and in three patients, lymph node metastases were observed. No differences in histopathological characteristics were observed between the FAP-related and sporadic carcinomas.

Comparative Genomic Hybridization

Chromosomal imbalances were detected in the majority of the FAP-related carcinomas (4/5). The mean number of changes was 3.8 (range 0–9). The mean number of gains (2.2; range 0–4) did not differ from the DNA copy number losses (1.6; range

Table 1 Histological characteristics of sporadic and familial adenomatous polyposis-related tumors

ID	Sex	Age	Type	Differentiation	Growth pattern	Peritumoral inflammation	Intratumoral inflammation	Fibroblastic reaction	T	N	Tumor diameter (cm)	Ampullary region
F1	F	50	Adeno	Poor/moderate	Circumscript	±	±	±	T4	N1		Yes
F2	M	58	Adeno	Moderate	Circumscript	±	++	+	T3	N1	12 cm	No
F3	M	41	Adeno	Poor	Diffuse	–	–	±	T4	N0		No
F4	M	55	Adeno	Moderate	Circumscript	+	+	±	T1	NX		No
F5	M	65	Adeno	Poor	Diffuse	±	±	++	T3	N1	> 2 cm	No
S1	M	78	Adeno	Poor	Diffuse	±	±	+	T2	N0	5 cm	No
S2	M	69	Muc		Circumscript	±	±	±	T4	N0	2	No
S3	F	76	Adeno	Poor	Diffuse	±	±	++	T4	N1		No
S4	M	73	Adeno	Poor	Budding	+	±	+	T4	N1	4	No
S5	M	79	Adeno	Moderate	Circumscript	±	±	+	T4	N0	13	No
S6	M	60	Adeno	Moderate	Circumscript	++	±	+	T3	N0		Yes
S7	M	25	Adeno	Poor	Circumscript	+	+	+	T3	N0	9.5	Yes
S8	M	32	Adeno	Poor	Diffuse	+	+	++	T3	N0	5	No
S9	F	72	Adeno	Well	Diffuse	±	±	+	T4	N1	5	Yes
S10	F	68	Adeno	Well	Circumscript	±	±	++	T3	N0	7.5	No

Sex: F, female; M, male. Type: Adeno, adenocarcinoma; Muc, mucinous carcinoma. Peritumoral inflammation, intratumoral inflammation and fibroblastic reaction: –, absent; ±, little; +, moderate; ++, extensive.

0–4). For the sporadic carcinomas, 9 of 10 tumors showed DNA copy number changes with an average of 4.6 changes (range 0–10) per tumor. The number of chromosomal gains was significantly higher than the losses 3.4 (range, 0–8) vs 1.2 (range 0–5), respectively ($P=0.03$). The chromosomal gains and losses are listed in Table 2 whereas Figure 1 shows a schematic presentation of the chromosomal imbalances detected in the FAP-related and sporadic carcinomas.

In the FAP-related carcinomas, chromosomal gains were detected in regions on chromosome 7 ($n=1$), 8 ($n=2$), 11 ($n=1$), 14 ($n=1$), 17 ($n=2$), 18 ($n=1$), 19 ($n=2$) and 20 ($n=1$) whereas losses were observed on chromosome 1 ($n=1$), 4 ($n=1$), 10 ($n=1$), 11 ($n=1$), 15 ($n=2$), 17 ($n=1$) and 18 ($n=1$).

In the sporadic carcinomas, gains most frequently (30% of the tumors) involved chromosomes 8, 12, 13 and 20. For these chromosomes the common regions of overlap were 8q11–13, 8q24–qter, 12p, 12q11–q21, 13q and 20q. Clear copy number gains (ratio >1.4) involved 13q, 12p and 5p11–13. High copy number amplifications (ratio >1.6) were seen at 8q24–qter and 20q. Genetic losses were observed in regions on chromosome 2 ($n=1$), 6 ($n=2$), 8 ($n=1$), 9 ($n=2$), 15 ($n=1$), 17 ($n=2$), 18 ($n=2$) and 21 ($n=1$).

A significant difference in chromosomal imbalances between the FAP-related and sporadic tumors was seen on chromosome 12. In 6/10 of the sporadic tumors, gains in regions of this chromosome were observed, whereas no gains were detected in the FAP-related carcinomas ($P=0.04$). Also for chromosome 13, genetic gains (even a clear copy) were seen in the sporadic carcinomas but not in the carcinomas of patients with FAP ($P=0.17$).

Methylation-Specific Multiplex Ligation-Dependent Probe Amplification

For both *immunoglobulin superfamily genes member 4 (IGSF4)* and *TIMP metallopeptidase inhibitor 3*

(*TIMP3*), hypermethylation was found in 3/5 FAP-related carcinomas and 1/10 of the sporadic carcinomas ($P=0.08$). *Estrogen receptor 1 (ESR1)* showed hypermethylation in four FAP-related carcinomas and in five sporadic carcinomas ($P=0.29$). Two FAP-related carcinomas and two sporadic carcinomas showed hypermethylation in the *APC* and in *H-cadherin (CDH13)* genes ($P=0.41$). A significant difference was observed between the FAP-related and sporadic carcinomas for the methylation of the *paired box gene 6 (PAX6)*. Hypermethylation of *PAX6* was observed in 3/5 FAP-related carcinomas vs no hypermethylation in the sporadic carcinomas ($P=0.02$) (see Figure 2). Hypermethylation of *glutathione S-transferase Pi (GSTP)* and *mutL homolog 1 (MLH1)* was observed in one sporadic tumor. In one FAP-related tumor, hypermethylation of *checkpoint with forkhead and ring finger domains (CHFR)* and *retinoblastoma 1 (RB1 b)* was seen. The average number of hypermethylated genes was 4 ± 3 in FAP-related carcinomas vs 1 ± 1 in the sporadic carcinomas ($P=0.08$). The following probes performed less reliable when using DNA isolated from formalin-fixed and paraffin-embedded tissue and are therefore excluded from further analysis: *CDKN2B* (ME001), *MGMT*, *WT1*, *ASC*, *STK11*, *GATA5*, *ESR1*, *PAX5A* and *CHFR* (all from ME002).

Discussion

The genetic pathways leading to the development and progression of small bowel carcinomas are not well characterized. Sporadic carcinomas develop infrequently in the duodenum, while it is the main site for malignant extra-colonic manifestations in patients with FAP. This study compares chromosomal and methylation alterations in duodenal FAP-related and sporadic carcinomas. To reveal similarities or differences in the pathways leading to these cancers, the data are also compared to literature data on colorectal and gastric cancer.

Table 2 Chromosomal imbalances as identified by CGH present in sporadic and familial adenomatous polyposis-related tumors

ID	CGH gains	CGH losses	No. of changes
F1	7q21–qter, 14q, 17q11–23, 18q11–12	10q, 15q, 17p, 18q12–qter	8
F2	8q23–qter, 11q11–13, 17q11–21, 19, 20	1p11–32, 4, 11q14–qter, 15q	9
F3			0
F4	8		1
F5	19q32–qter		1
S1	6p11–12, 12q14–21, 13q, 15q24–qter	2q21–24, 21	6
S2	12q13–15, 20		2
S3	7pter–q22, 8q (8q24–qter high copy), 13q (clear copy), 22	8p, 9p, 15q, 17p, 18	9
S4	8q, 13q	9p, 17q23–qter	4
S5			0
S6	1q23–qter, 5p, 16p, 19, 20q		5
S7	12p (clear copy), 17	18p	3
S8	12		1
S9	3p11–14, 6p11–21, 12q13–15, 12p, 14q12–21, 15q24–qter, 18p, 20q (high copy)	6p21–pter, 6q	10
S10	8q11–13, 8q23–qter, 5p11–13 (clear copy), 12q14–15, 17q22–qter, 20q		6

F, FAP related tumors; S, sporadic tumors.

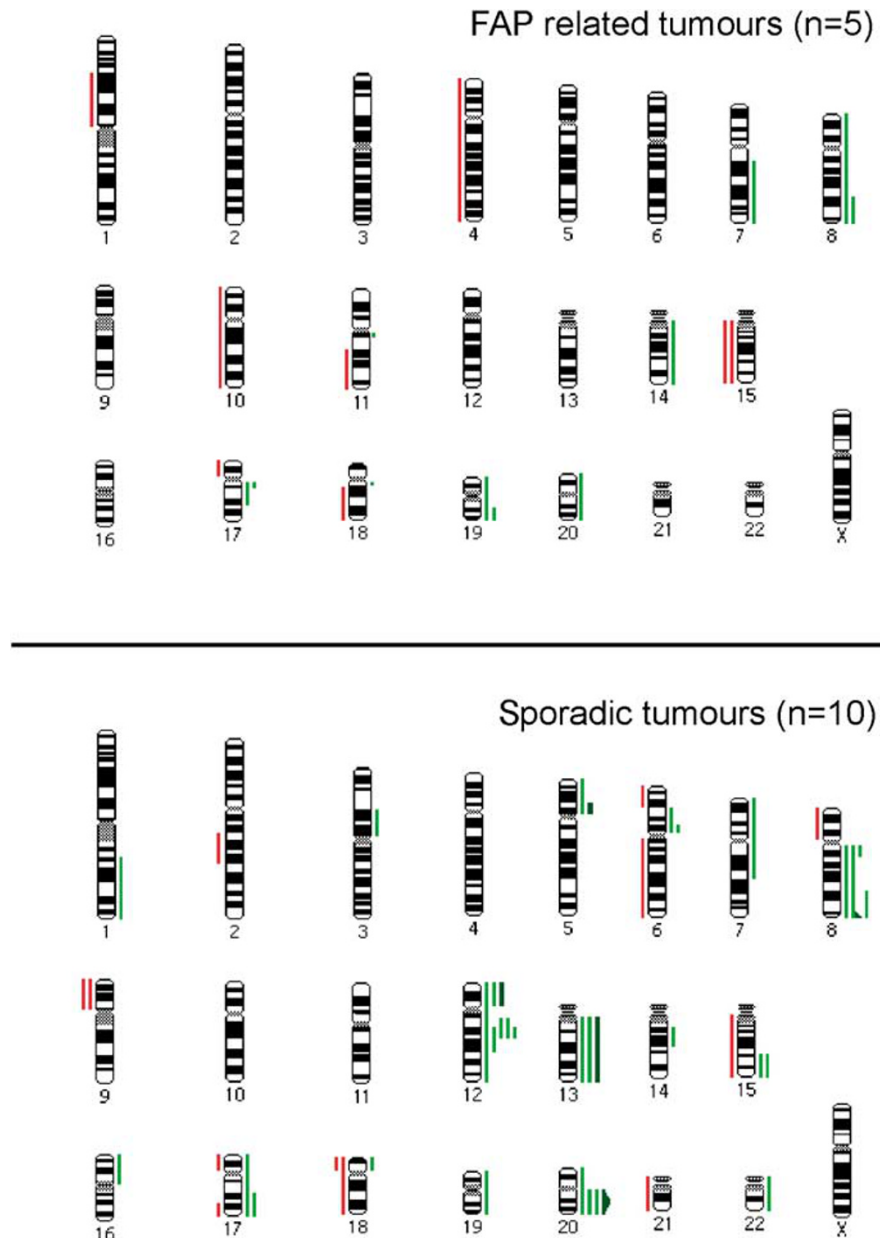


Figure 1 Summary of all chromosomal imbalances detected by CGH in sporadic and familial adenomatous polyposis-related duodenal tumors. Lines on the left and right of the chromosomes indicate respectively losses (red) and gains (green). A thin line indicates genetic aberrations crossing the 0.8 or 1.2 threshold, while clear copy changes crossing the 0.6 and 1.4 are indicated by a thick line (dark green). High copy changes, indicated by a ratio larger than 1.6 are indicated by an additional spot on a line (dark green).

We observed hypermethylation in several well-known tumor suppressor genes in the sporadic duodenal carcinomas. In accordance with the data in our study, a similar frequency of hypermethylation of *TIMP3* (10%) was seen in colorectal carcinomas.^{34,36} In contrast, a higher frequency of hypermethylation was found in colorectal carcinomas for *APC* (± 50 vs 20% in our study)^{34,36} and for *CDH13* (65 vs 20% in our study).³⁷ In addition, frequent hypermethylation of the genes *cyclin-dependent kinase inhibitor 2A* (*CDKN2A*)^{38,39} and *Ras association domain family 1* (*RASSF1A*)^{40,41} was found in colorectal tumors, in contrast, no hyper-

methylation was found in our study. Almost no hypermethylation was found in the promoter region of the *PAX6* gene in colorectal carcinomas.⁴² For gastric cancer, the frequencies of hypermethylation of *APC* and *TIMP3* were higher compared to our findings (± 78 and $\pm 43\%$, respectively). Similar to colorectal cancer, *CDKN2A* and *RASSF1A* are also frequently hypermethylated in gastric cancer in contrast to the duodenal carcinomas studied here.⁴³ Although, the MS-MLPA and the conventional MS-PCR showed very similar results in our hands for the *O-6-methylguanine-DNA methyltransferase* (*MGMT*) gene,³⁵ differences found in comparison

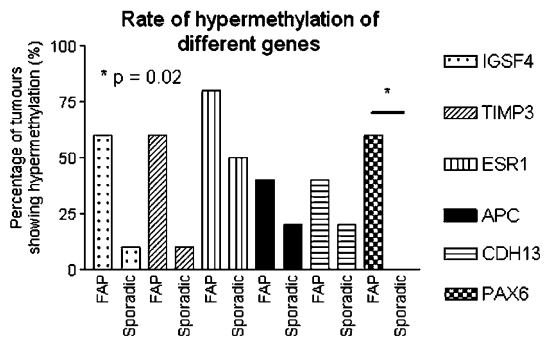


Figure 2 Percentage of sporadic and familial adenomatous polyposis-related duodenal tumors showing hypermethylation in the genes *IGSF4*, *TIMP3*, *ESR1*, *APC*, *CDH13* and *PAX6*.

with literature data may arise from the different methods.

Several genomic imbalances in duodenal carcinomas were detected by CGH. Similar to what has been described for colorectal carcinomas; sporadic duodenal tumors showed frequent gains on chromosomes 8q, 12p, 13q and 20q. In contrast to sporadic colorectal cancer, only one loss at 18q was observed^{44–47} in the duodenal carcinomas. In gastric carcinomas, again frequent gains involved 8q, 13q and 20q but only occasionally 12p.^{48,49} In contrast to both colorectal and gastric carcinomas, sporadic duodenal carcinomas often showed gains in the region 12q13–21. Interestingly, a difference was observed between sporadic and FAP-related tumors with respect to copy changes on chromosomes 12 and 13 as detected by CGH. Gains were only present in the sporadic tumors and not in the FAP-related tumors. Chromosomal gains on chromosome 12p were also observed in pancreatic^{50,51} and gastric carcinomas.⁴⁹ Recently, gains of 12p were shown to be late events in liver metastases of colorectal carcinomas, indicating their role in tumor progression.⁴⁶ Likewise, gains of 12p were often observed in the advanced stages of a wide variety of tumors.^{46,52,53} In the current study, most carcinomas also presented at an advanced stage. However, since the tumor stage was comparable in both groups, this could not explain the difference detected between sporadic and FAP-related carcinomas. Possible candidate genes on chromosome 12 include *Kirsten rat sarcoma viral oncogene homolog (KRAS2)*, *cyclinD2*, *MDM2* and (*wingless-type mmtv integration site family (WNT1)*). In addition, gains at 13q as observed here in sporadic duodenal carcinomas, were shown frequently in both colorectal^{44,45,47,54} and gastric carcinomas.^{48,49} Also a difference was observed in the hypermethylation status of the *PAX6* gene between sporadic and FAP-related duodenal carcinomas. *PAX6* is a highly conserved transcription factor, which plays an important role in the normal embryological development.⁵⁵ Furthermore, this transcription factor is implicated in pancreatic and intestinal endocrine cell fate determination and in eye and brain development.⁵⁶ However, the role of

PAX6 in the development of intestinal carcinomas is less clear and also the difference in hypermethylation of *PAX6* between FAP- and sporadic carcinomas is difficult to explain. Future research must further elucidate the exact role of *PAX6* in the development of duodenal carcinomas.

The mutations in the *APC* gene (located at chromosome 5q21–22) often found in patients with FAP lead to a disturbed function of the *APC* protein. The key tumor suppressor function of *APC* is to regulate the stability and cellular localization of β -catenin.⁵⁷ β -Catenin is a bifunctional protein with a crucial role in cell–cell adhesion⁵⁸ and a signaling role in the Wnt pathway.⁵⁹ Loss of functional *APC* leads to a disturbed Wnt signaling pathway, which is involved in many types of cancer⁶⁰ and aberrant activation of this pathway could possibly result in chromosomal instability as found in colon cancer.⁶¹ The genes of *WNT1* and *WNT10B*, both members of the Wnt gene family and encoding for Wnt signaling proteins, are located in the chromosome 12q13 region.⁶² Interestingly, in three sporadic carcinomas gains are found in this region. Given the importance of Wnt signaling in tumor formation, this may suggest that the chromosomal imbalances on chromosome 12 in the sporadic carcinomas are associated with Wnt signaling abnormalities. One has to realize that Wnt signaling is already disturbed in FAP-related carcinomas, which may explain the absence of chromosomal aberrations in this region of chromosome 12 here. So although different aberrations are detected in FAP-related (*APC* mutations) and sporadic (+12q) tumors, their effects may be similar.

For FAP-related carcinomas, there are only karyotyping studies of colonic adenomas,⁶³ colonic carcinomas⁶⁴ and desmoid tumors.⁶⁵ In comparison to CGH, a disadvantage of karyotyping is that it requires the culture of fresh tumor cells, which can only be achieved for some of the (malignant) tumors and which may introduce culture artifacts such as clonal selection.²⁴ Since macro-dissected tumor tissue was used in this study, almost only tumor DNA was used for the CGH, thus increasing the accuracy of the measurements. However, carcinomas used in this study were routinely processed, formalin-fixed and paraffin-embedded. DNA isolated from paraffin-embedded tissue is often degraded, with the bulk of the DNA showing a fragmented size. This could complicate the nick translation for the CGH and may lead to inferior results when compared to results obtained by CGH after using frozen tissues. Therefore, some chromosomal imbalances could have been left undetected in the paraffin-embedded carcinomas investigated here, especially in the older specimens.

The karyotyping study of desmoid tumors in patients with FAP mainly showed a defect on chromosome 5q, where the *APC* gene is located.⁶⁵ The FAP-related adenocarcinomas investigated here showed no abnormalities in this region. However,

small deletions present in the *APC* gene could be undetected by CGH. Interestingly, we found hypermethylation of the *APC* promoter region in two FAP-related carcinomas, suggesting biallelic inactivation of the *APC* gene. To our knowledge, in the only study described so far which investigates a colon carcinoma of a patient with FAP, no abnormalities on chromosome 12 were observed and that is in accordance with our results.⁶⁴ Sporadic colon carcinomas show gains of 12p in approximately 30% of the cases.⁴⁴

Both sporadic and FAP-related carcinomas showed genetic gains on chromosome 8 (especially 8q). Diep *et al*⁴⁶ suggested that gains of 8q could be involved in establishing distant colorectal metastases. Gains of 20q, which may be an early change in both primary colorectal carcinomas and their liver metastases, were also seen in sporadic and FAP-related carcinomas. Furthermore, the frequency of gains of 20q was reported to increase with Dukes' stages, emphasizing their role in tumor progression as well.^{46,54} In addition, frequent gains of 20q have been reported in other tumors, including gastric adenocarcinomas.⁶⁶

The current study showed no differences in chromosomal imbalances between tumors in the ampullary region and more distally located tumors, probably due to small numbers. In accordance with Chang *et al*,²¹ several gains were found in sporadic tumors located in the ampullary region, such as on chromosomes 1q, 3p, 12p, 14q, 18p and 20q. In contrast to Blaker *et al*,²⁰ who reported frequent losses of 18q in sporadic small bowel adenocarcinomas, only one case of loss of chromosome 18 and one loss of chromosome 18p were observed in this study. The study of Blaker *et al*²⁰ however, included only four duodenal carcinomas, of which two tumors showed loss of chromosome 18 (q). The two only studies describing hypermethylation of promoter regions of genes in small bowel carcinomas also showed both similarities and differences to our study. Brücher *et al*²² demonstrated the same frequency in hypermethylation of *APC*, however differences were observed for the prevalence of hypermethylation of *mutL homolog 1 (MLH1)* and *CDKN2A*. These differences may have been caused by our smaller study population. In comparison with results of Kim *et al*,²³ the same high frequency of hypermethylation was found here for *EST1*, although *MLH1* and *CDKN2A* also differed from our study.

In summary, the chromosomal and methylation alterations of 15 duodenal carcinomas were analyzed in this study. The results suggest that gains on chromosomes 8, 17 and 19 and losses on chromosome 15 might play a role in the development and/or progression of FAP-related carcinomas ($n=5$). In the sporadic carcinomas ($n=10$), frequent gains were seen on chromosomes 8, 12, 13 and 20. These findings are similar to what has been described for sporadic colorectal and gastric carcinomas. In con-

trast to results in sporadic colorectal cancer, only once was a loss on 18q observed in sporadic duodenal carcinomas. Furthermore, in contrast to both colorectal and gastric carcinomas, sporadic duodenal carcinomas often showed gains in the region 12q13–21. In addition, although the numbers of carcinomas studied here are small, different patterns of chromosomal imbalance could be detected in sporadic vs FAP-related carcinomas. Gains at chromosome 12 were not observed at all in duodenal carcinomas of patients with FAP, suggesting that identification of the genes in 12q13–21 could lead to a better understanding of the carcinogenesis pathways in both sporadic as well as FAP-related duodenal carcinomas. Interestingly, *APC* mutations present in patients with FAP may have a similar effect on the Wnt signaling as gains at 12q in sporadic tumors, suggesting that even though the aberrations detected may differ, a similar pathway is affected. Furthermore, methylation of multiple CpG-islands is present in both sporadic and FAP-related duodenal carcinomas whereas the methylation status of *PAX6* seems to be different in FAP-related carcinomas compared to sporadic carcinomas.

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