Chromosomally and microsatellite stable colorectal carcinomas without the CpG island methylator phenotype in a molecular classification

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Abstract. We hypothesized that in a comprehensive analysis of colorectal carcinomas (CRC) the three currently known major molecular mechanisms of carcinogenesis (i.e., chromosomal instability, microsatellite instability, and CpG island methylator phenotype, CIMP) would associate with the molecular features indicative of these pathways, allowing a molecular classification. A prospectively collected clinicopathologically well-characterized series of 130 CRCs was tested for chromosomal instability (DNA-flow cytometry and analysis of allelic imbalance with microsatellite markers 5q21, 8p21, 9q21, 17p13, and 18q21), microsatellite instability (Bethesda panel), CIMP (MethyLight), and mutations of K-ras, B-raf, APC, and p53. Morphology was reviewed, and nuclear ß-catenin translocation was assessed by immunohistochemistry. Based on the molecular features, sporadic high-degree microsatellite instable tumours, tumours of the hereditary non-polyposis coli carcinoma syndrome, and 'sporadic standard-type' CRC could be delineated (14, 4, and 55, respectively). However, overlap between classes was seen for 46 of the remaining tumours where widespread or occasional methylations (excluding MLH1) were observed, and the majority had chromosomal instability. Importantly, a group of 11 tumours was observed without either microsatellite or chromosomal instability, nor any methylation. Morphologically, these tumours were without any distinguishing features, all had tumour budding and 10 showed nuclear ß-catenin translocation. Overall, the data give an overview of the molecular classes in CRC that should be taken into account in studies on carcinogenesis and clinicopathological studies. Specifically, the absence of CIN, MSI, and CIMP in an 8.46% fraction of tumours delineates a group to be aware of.

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Introduction

In the classical work by Vogelstein *et al*, colorectal carcinoma (CRC) carcinogenesis was shown to be associated with frequent losses of whole chromosomes or parts thereof (1). It is recognized that acquired defects of cellular control mechanisms (e.g., DNA strand repair, kinetochore function, chromatid segregation) lead to sustained instability of the genome in tumour cells and progression of genomic changes (2). Chromosomal instability (CIN) is thought to cause loss of tumour suppressor genes (TSGs). Thus, CIN has largely become equated with the suppressor pathway of CRC (3). Technically, CIN can be assessed by DNA-flow cytometry or comparative genomic hybridization that both reflect gross alterations, or assays for allelic imbalance (AI) for more subtle alterations. In CRC loci at 5q21, 8p21, 9p21, 17p13, and 18q21 are targeted most frequently.

Even in the initial publication by Vogelstein *et al* (1) unexpected additional bands in the restriction fragment length analyses were noted. Very soon afterwards, this was recognized as high-degree microsatellite instability (MSI-H) and traced to an inherited loss of mismatch repair enzymes in the hereditary non-polyposis (HNPCC) syndrome, defining the so-called mutator pathway as an alternative pathway of CRC carcinogenesis (4). By consensus, MSI-H is best assayed with the Bethesda panel of microsatellite markers (5).

As a third major molecular mechanism, high-degree promoter methylations in CpG islands were discovered later, defining the CpG island methylator phenotype (CIMP) (6). There is consensus that quantitative analyses of methylations are best for determination of CIMP, and two marker panels have been published (7,8). As is well recognized now, epigenetic silencing of the mismatch repair gene MLH1 is responsible for MSI-H outside the HNPCC syndrome (9). Many of these tumours can also be distinguished morphologically (10).

In addition to these molecular aberrations that reflect the major pathways of carcinogenesis, point-mutations of the p53 gene, and the K-Ras or the B-Raf genes are characteristic

findings in CRC. These mutations are known to interfere with apoptosis or signal transduction. Furthermore, mutation of the adenomatous polyposis coli (APC) gene is a long-recognized, classical molecular feature of CRC. APC gene mutation is known to interfere with wnt-signalling, and this wnt-dysregulation is characterized by translocation of ß-catenin to the nucleus (11). Recently, in a very large study (12) the 'genomic landscapes' of CRC have been delineated: it has become clear that each CRC harbours a limited number of mutations (average about 15 each) from a set of 139 'candidate cancer genes'.

Surprisingly, even though extensive research has thus been devoted to the different pathways of CRC carcinogenesis, and even though a classification of colorectal cancer according to clinical, morphological and molecular features has been suggested by Jass, based on data compiled from different series (13), to our knowledge there has been no study that addressed how the molecular and morphological features indicative of each of these pathways are distributed, if in a larger series each tumour is assayed for all of the molecular changes. In this study, we carried out a comprehensive characterization of a prospectively collected consecutive series of 130 clinicopathologically well defined surgical CRC specimens. We addressed if currently recognized pathways of CRC carcinogenesis would be mirrored in the molecular features of the tumours, and if this would allow a molecular classification. Additionally, morphological associations were explored.

Materials and methods

Specimen collection, morphological studies and immunohistochemistry. A consecutive series of 130 CRCs was collected in the years 2002-2006. Resection specimens (ordinary adenocarcinomas or mucinous carcinomas; no neoadjuvant treatment) were received fresh from surgery. Cubes of about 3 mm^3 were cut from the invasive margin and snap-frozen in liquid nitrogen. Normal mucosa was taken from near the resection margins. Patients had given informed consent, and the study was approved by the local ethics committee.

After fixation in buffered formalin the specimens were dissected and reported by one of the authors (Friedrich Prall) with typing, grading and staging according to UICC TNM. Tumours were blocked generously, and at least one block was taken also from the margins. Information on clinical staging and patient's personal and family history (as recorded on first-contact interviews) were extracted from the clinical charts.

In the course of the study the slides of all tumours were reviewed twice. First, all slides were reviewed without knowledge of the molecular classes. The invasive margins of the tumours were typed as expansive vs. infiltrative, and tumour budding and nuclear ß-catenin translocation were assessed using pan-cytokeratin and ß-catenin immunostain, respectively, as detailed before (14). Furthermore, loss of MLH1 and MSH2 expression was tested by immunhistochemistry.

Slides were also scrutinized for cyto- and histomorphological features of serration as described by Tuppurainen *et al* (15). This was sought for in the central parts of the tumours as well as in their marginal components. Furthermore, presence of a residual adenomatous component was recorded, and the tumours were assessed for a villous component (absent, 10-50%, >50%, corresponding to villous adenocarcinoma) as described by Loy and Kaplan (16).

The second slide review was done to search for histomorphological differences between the molecular classes. Knowing to which class a given tumour had been assigned to, slides were looked at with attention to details that would either not be noted by the above criteria of WHO typing and grading, as well as serration and villosity; or would only be noted in synoptic viewing as an unusual combination of the above features.

DNA extraction and molecular studies. For extraction of DNA, frozen sections were taken from the snap-frozen material to ascertain in initial sections that the tumours were well represented, and subsequent sections were digested in proteinase K (200 μg/ml) overnight at 56˚C. Genomic DNA was extracted by means of the NucleoSpin Tissue Kit (Machery-Nagel, Düren, Germany) according to the manufacturer's protocol.

The molecular analyses were done as previously published (14,17). Cases were classified as MSI-H if two or more markers of the Bethesda panel of microsatellite markers showed band shifts. AIs were examined using the following dinucleotide markers: D5S1385 (5q21); D8S1734, D8S1771, NEFL (8p21); D9S942, D9S1748 (9p21); D17S1832, D17S250 (17p23); and D18S70 (18q23). AI was scored positive if the tumour/normal ratios were <0, 5 or >2, 0. K-Ras codon 12 and 13, p53 (exons 5-8), and APC gene mutations (mutation cluster region in exon 15, codons 1260-1547) were tested as before (14,17). B-Raf V600E mutation analysis was performed as described by Samowitz *et al* (18). PCR products were purified, cycle-sequenced and analyzed with a 3100-Avant Genetic Analyzer (Applied Biosystems).

Methylations were assessed by quantitative real-time PCR using the MethyLight technology as published by Ogino *et al* (7). The panel consisted of the following markers: CACNA1G, CDKN2A, CRABP1, MLH1, NEUROG. COL2A1 was used for normalization of the input DNA. The EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) was used for bisulfite treatment of genomic DNA. A locus was classified as methylated when the percentage of methylated reference exceeded 4 (PMR >4).

DNA-flow cytometry was done as described by Hedley *et al* (19). For each tumour the paraffin-block was selected that contained the maximum amount of neoplastic tissue. Two 30-μm thick paraffin sections were pooled and paraffin was removed by xylene treatment. Samples were rehydrated and nuclei were released by pepsin treatment (30 min at 37˚C in 1 ml 0.5% pepsin in 0.9% NaCl, pH 1.5). Pepsin solution was removed and nuclei were resuspended in 500 μl PBS. Propidium iodide was added to a final concentration of 10 μg/ml. Flow cytometry was performed on a FACSCalibur [Becton-Dickinson (BD), Heidelberg, Germany] taking advantage of the CellQuest® (BD) software. Tumours were scored as diploid if an unequivocal single DNA stem line was observed in the DNA histograms, and cases with two

Table I. Associations of molecular classes with clinical and morphological features.

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	P-value[a]
Gender								0.142
Male	4	5	16	29	6	2	2	
Female	10	6	9	26	4	9	2	
Site								0.0001
Right colon	14	4	8	13	4	3	4	
Left colon/rectum	0	7	17	42	6	8	0	
Age (years)								0.03
<50	0	0	1	3	1	1	1	
50-75	9	8	21	43	8	3	3	
>75	5	3	3	9	1	7	0	
UICC stage								0.214
Stage I	6	2	8	8	0	2	1	
Stage II	2	2	6	15	2	6	2	
Stage III	4	4	4	18	5	3	0	
Stage IV	2	3	7	14	3	0	1	
Grade								0.086
G3	7	3	8	11	2	0	2	
G1/G2	7	8	17	44	8	11	2	
Mucin								0.0001
With mucin[b]	11	3	5	7	1	3	2	
Without mucin	3	8	20	48	9	8	2	
Villousity								0.12
Absent	11	11	23	53	10	9	3	
Present (10-50%)	3	0	2	2	0	2	1	
Villous adenocarcinoma	0	0	0	0	0	0	0	
Serration								0.066[c]
Present	3	0	0	1	0	0	0	
Absent	11	11	25	54	10	11	4	
	14	11	25	55	10	11	4	

[a]χ^2 test. [b]Mucinous carcinoma (>50%), or mucinous component 10-50%. [c]Fisher's exact test.

major DNA stem lines were classified as aneuploid. In a fraction of tumours, the DNA histograms remained equivocal. On the rationale that a false-positive call for diploid status in particular should be avoided such cases were classified as undetermined/equivocal (Fig. 1).

Statistical analysis. All data were entered into a computerized data bank (Statistical Package for the Social Sciences, SPSS version 13.0). Cross tabulations with χ^2 significance testing were used as implemented with this software.

Results

A synopsis of the clinical and morphological data of this series of CRCs and their associations with molecular classes can be gleaned from Table I.

Classification by molecular findings. Based on the heuristic hypothesis that for a given CRC its pathway of carcinogenesis is reflected in molecular features the molecular data were used to delineate seven groups of tumours. Classifications

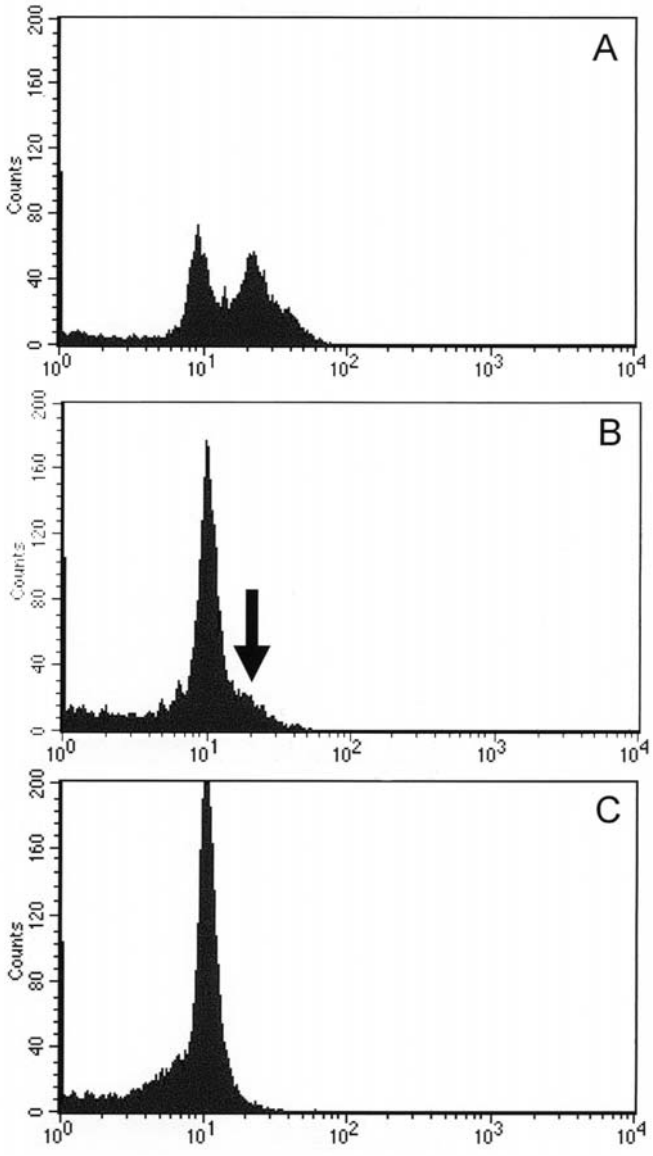

Figure 1. Examples of DNA histograms classified as: (A), aneuploid; (B), equivocal; and (C), diploid. Note the rudimentary peak (arrow) recorded for the case in (B), that precluded a definite classification.

were made as set out in the flow-chart in Fig. 2, and the full set of the molecular data is presented in Fig. 3.

A group of 14 tumours (group 1 in Fig. 3) showed MSI-H, and had methylations with two or more of the markers. Methylations in most cases were strong and included methylation of MLH1, and loss of MLH1 expression was seen by immunohistochemistry. In one case MLH1 methylation was clearly detectable but did not exceed a PMR of 4; however, this tumour showed loss of MLH1 expression. B-Raf gene mutations were seen in ten cases, and K-Ras gene mutations in three. Using microsatellite markers, AI as evidence of CIN was not seen in any of these tumours, DNA-aneuploidy was recorded in three cases, however. This type of tumour is well recognized as sporadic MSI-H CRC (spMSI-H), and it could be separated in a straightforward fashion.

A group of 11 tumours (group 2 in Fig. 3) showed strong methylations with at least three of the markers used. In spite of a methylator phenotype, however, MLH1 methylation and loss of expression was not a feature of these tumours, and, accordingly, for these tumours MSI-H was not observed. B-Raf or K-Ras gene mutations were fairly frequent among these (3 B-Raf, 4 K-Ras mutations); in fact, the only B-Raf gene mutations outside group 1 were found in this group. Notably, though sharing CIMP-high with the tumours in group 1, AI was observed for eight of the 11 tumours in group 2. These tumours correspond to 'CIMP-high, non-MSI-H tumours' previously described in the literature.

The largest group (group 4 in Fig. 3; N=55) was characterized by the combination of CIN (demonstrated by DNA-aneuploidy and/or AI), and absence of MSI-H as well as methylations. These molecular features were considered compulsory to make the assignment. In addition, nuclear ß-catenin translocation, and APC as well as p53 gene mutations were frequent among these (43, 35, and 36 tumours, respectively). Tentatively, these can be named 'standard type sporadic CRC'.

Four tumours (group 7 in Fig. 3) showed molecular features of CRCs associated with the HNPCC syndrome, even though the family histories of these patients were not suggestive of the syndrome. Specifically, MSI-H was observed, but methylations and CIN were absent. Nuclear ß-catenin translocation was found in one tumour, APC as well as p53 gene mutations were not observed in any of the tumours. Loss of MLH1 expression could be demonstrated in one case, but neither loss of MLH1 nor of MSH2 was seen for the remaining three cases. All tumours were located in the right colon, patients were comparatively young (41, 55, 60, and 69 years).

Tumours were observed (groups 3 and 5 in Fig. 3) that because of the lack of MSI-H clearly did not belong to groups 1 or 7. Though not meeting the criteria fully, these tumours shared many features with group 4 tumours (the standard type sporadic CRCs): on one hand, there were tumours (group 3; N=25) that mostly were CIN and had methylations at one or two loci, thus placing them between groups 2 and 4; on the other hand, there were tumours (group 5; N=10) without any methylation for which definite evidence of CIN could not be gained, accordingly, this latter group of tumours was placed between groups 4 and 6. Thus, these tumours were labelled 'indeterderminate tumours, types 1 and 2', respectively.

Finally, as an unexpected finding, there were 11 tumours for which none of the major molecular findings of the known pathways could be ascertained (group 6 in Fig. 3). Specifically, they lacked MSI-H, AI or DNA-aneuploidy, and methylation. Frequencies of APC gene mutations were low (30%), but p53 and K-Ras gene mutations were as common as in groups 2-5. Notably, ß-catenin translocation was present in all but one case. As the pathway to carcinogenesis for these tumours is not clear at present, the tentative designation of 'X-type CRC' was applied to these.

Taken together, the initial hypothesis was tested positive for the main groups of CRC, i.e., the standard-type CRCs (42.31%), the HNPCC type tumours (3.08%), the sporadic MSI-H tumours (10.77%), and the CIMP-H/non-MSI-H tumours (8.46%). Cutting across these main groups there were tumours most closely related to the standard-type CRC ('indeterminate tumours types 1 and 2'; 8.46%, and 19.23%,

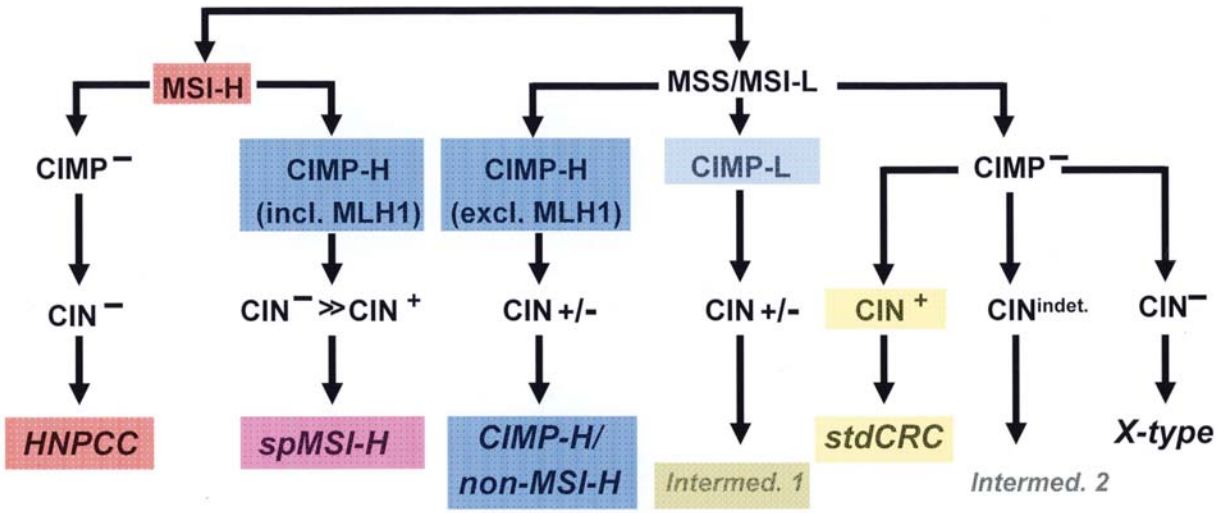

Figure 2. Flow-chart illustrating the approach to classify the tumours investigated in this series based on the major molecular data, i.e., MSI, degree of methylations, and CIN. Additional molecular and immunohistochemical data (mutations of the K-ras, B-raf, p53, APC genes, and ß-catenin nuclear translocation) were not used for the classifications, but showed characteristic associations with some of the molecular classes (see Fig. 3 for the full set of molecular data).

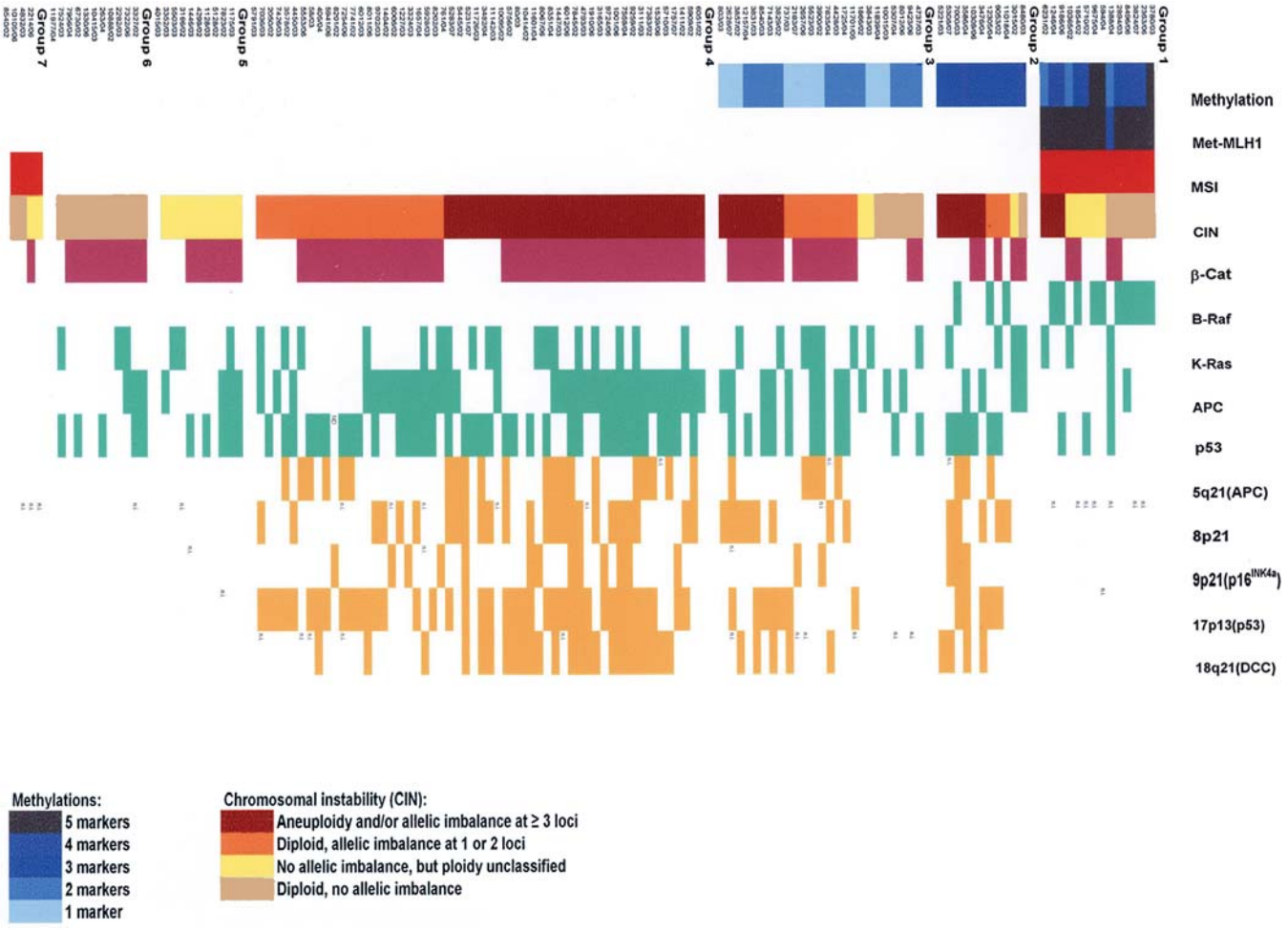

Figure 3. Heatblot summarizing the molecular data. Cases are arranged in molecular classes according to the presumed pathways of carcinogenesis. The 1st row gives the ID numbers of the cases. Numbers of methylation markers positive are in black to blue as indicated (bottom). Type of chromosomal instability was classified as indicated (bottom). Rows in yellow represent the results of the microsatellite analyses for AI at the different loci indicated; n.i. if none of the markers used was informative.

respectively). And finally, there were 'X-type tumours' (8.46%) that did not comply with the initial hypothesis.

Morphological associations. Associations of the molecular classes of CRCs delineated above with morphological

features are summarized in Table I. SpMSI-H tumours were clearly separated from the rest. As a salient feature, these tumours had serration in three cases, and the remaining eleven were heteromorphous with a mucinous component and areas with trabecular or solid architecture. However, for tumours of the other groups no characteristic or distinguishing morphological features were observed.

Discussion

This study was carried out to test if by a comprehensive analysis of a large series of surgical CRC specimens of the three currently known major molecular mechanisms of carcinogenesis would consistently be reflected in the molecular features indicative of these pathways. In some aspects the results fit well with the current views on CRC carcinogenesis, but there are important deviations.

As expected spMSI-H tumours (group 1) and HNPCC-type tumours (group 7) were clearly set off from the rest. In these groups, the molecular features of the tumours corresponded well with the pathways of carcinogenesis ascribed to them. The group of 'standard type sporadic CRCs' (group 4) also appeared consistent with the theory. For these tumours CIN was documented, but MSI-H and CIMP were absent. However, taken together only these 73 of the 130 total of tumours investigated (56.1%) were observed to fall into the expected places (14, 4, and 55 in groups 1, 7, and 4, respectively). Apart from these, there was overlap between classes (groups 2, 3, and 5), and finally there was an unexpected group (group 6) that did not correspond at all with the current concepts. Tentatively, these were named 'X-type' CRC.

Based on CIMP, at first sight the tumours in groups 2 and 3 seem to be closely related to the spMSI-H tumours in group 1, absence of epigenetic MLH1 silencing making the difference. But, since the mutator pathway does not operate in these, the suppressor pathway would have to take its place by CIN, and indeed CIN was frequent among these. Thus, based on CIN, these tumours would appear to be related most closely to the 'standard type sporadic CRC' (group 4). In fact, as CIN was observed frequently in groups 2 and 3, it could be argued that CIMP would be relevant for CRC carcinogenesis only if the MLH1 gene promoter is affected, although global expression analyses have shown unique gene expression patterns for CIMP-high tumours without MSI-H (20).

The above hypothetical argument presumes the current view that either CIN or MSI-H has to be realized for CRC carcinogenesis. However, the finding of the 'X-type' CRC raises questions about this concept. In this group of 11 tumours (8.46% of the total), neither MSI-H nor CIN were seen. For each of these tumours AI was assayed negative at the archetypical loci, and a diploid DNA-status was demonstrated by flow cytometry. Notably, a conservative approach was followed in the interpretations of the DNA-histograms where a diploid DNA-status was scored for unequivocal histograms only, the residuum of equivocally aneuploid and equivocally diploid tumours being placed in the ad hoc default category of 'undetermined'. Furthermore, presence of sufficient tumour in the frozen material used for the assessment of AI and the paraffin-blocks used for DNA-flow cytometry was checked. Absence of any evidence of CIN or MSI-H in a non-negligible fraction of about 10% in this series are difficult to reconcile with the current view that CIN (by allelic loss or epigenetic silencing) or MSI-H are indeed the only moving forces of CRC carcinogenesis. In fact, it could even be argued that this group is even larger and should include the chromosomally stable, non-MSI-H tumours in groups 2 and 3 (1 and 6 in groups 2, and 3, respectively).

Minor fractions of CRCs without evidence of CIN or MSI-H have previously been reported in a limited number of studies by various analytical techniques. Georgiades *et al* addressed CIN and its heterogeneity within the primaries as well as its progression in xeno-transplants (21). DNA-flow cytometry and comparative genomic hybridization were employed to assess CIN. In this elegant study, they showed that among sporadic non-MSI-H CRCs there resided a fraction of about 20% (4 of 17) of diploid tumours with a very low number of chromosomal changes and without intratumoural heterogeneity. Interestingly, none of these grew after xeno-transplantation, but synchronous regional lymph node metastases were not consistently absent (3 Dukes C in this group). Small sample size, of course, was a limitation of that study.

Larger, consecutive series of 160 and 209 tumours were studied and reported later (22,23). In one study CIN was assessed by DNA-flow cytometry and by scoring AI using 11 dinucleotide markers (22), and 19% of the tumours were observed to combine diploid DNA-status and absence of AI with absence of MSI-H. In the other report (23), dinucleotide markers at 1p, 2p, 5q, 17p, and 18q were used to study AI, and a fraction of 11% of the tumours turned out to have neither MSI-H nor AI. Thus, lack of CIN and MSI-H was observed in frequencies similar to the results from the present study. In the present study, however, the tumours were investigated more comprehensively. Most importantly, CIMP was tested, and no evidence was found of epigenetic TSG silencing that otherwise could have been hypothesized to substitute for losses of TSGs by CIN. A morphological study, in addition, did not reveal any differences between the 'X-type tumours' and the tumours in groups 2- 4, and group 7; only the spMSI-H tumours could clearly be distinguished morphologically.

X-type' tumours could be an interesting object for further studies, particularly for single nucleotide polymorphism (SNP) assays using laser-capture microdissected neoplastic glands. On one hand, though less likely, it cannot be excluded completely that by the methods used in this study genomic aberrations have been missed for at least some of the tumours assigned to this group. In this case, 'X-type' tumours probably would not be truly different from the standard sporadic type colorectal carcinomas, although a different set of TSGs could be targeted. Alternatively, 'X-type' tumours could be set apart by a predominance of deletions or amplifications that involve circumscript parts of chromosomes only. In this case, the tumours presumably would have developed along a pathway analogous to the suppressor pathway, but 'background noise CIN' could be absent or considerably reduced. This could be an advantage when searching for TSG loci, the important question would then be if chromosomal regions with aberrations would fall into the 'hills' of the 'genomic landscapes' (12),

i.e., if they would coincide with candidate cancer genes. Finally, uniparental disomy that has been shown to occur in CRC (24,25) could be a distinguishing feature of 'X-type' tumours. In this case, the question would arise if perhaps specific mechanisms could be behind or if there are loci that are targeted preferentially.

In conclusion, the molecular and clinicopathological classification of the CRCs in this series clearly separated spMSI-H, HNPCC-type tumours, as well as standard-type CRCs with CIN. Non-CIMP, non-MSI-H tumours remained a less well defined class, most closely related to the standard-type. Most notably, as a group not previously appreciated in combined molecular and clinicopathological classifications (13) there was the group of 'X-type tumours' that had neither CIN, nor MSI-H, nor CIMP. This group may be of some interest for further genetic studies.

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