Tumorigenesis and Neoplastic Progression

Aneuploidy Arises at Early Stages of *Apc*-Driven Intestinal Tumorigenesis and Pinpoints Conserved Chromosomal Loci of Allelic Imbalance between Mouse and Human

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Although chromosomal instability characterizes the majority of human colorectal cancers, the contribution of genes such as adenomatous polyposis coli (*APC*), *KRAS*, and *p53* to this form of genetic instability is still under debate. Here, we have assessed chromosomal imbalances in tumors from mouse models of intestinal cancer, namely *Apc*^{+/^{1638N}}, *Apc*^{+/^{1638N}}/*KRAS*^{V12G}, and *Apc*^{+/^{1638N}}/*Tp53*^{-/-}, by array comparative genomic hybridization. All intestinal adenomas from *Apc*^{+/^{1638N}} mice displayed chromosomal alterations, thus confirming the presence of a chromosomal instability defect at early stages of the adenoma-carcinoma sequence. Moreover, loss of the *Tp53* tumor suppressor gene, but not *KRAS* oncogenic activation, results in an increase of gains and losses of whole chromosomes in the *Apc*-mutant genetic background. Comparative analysis of the overall genomic alterations found in mouse intestinal tumors allowed us to identify a subset of loci syntenic with human chromosomal regions (eg, 1p34-p36, 12q24, 9q34, and 22q) frequently gained or lost in familial adenomas and sporadic colorectal cancers. The latter indicate that, during intestinal tumor development, the genetic mechanisms and the underlying functional defects are conserved across species. Hence, our array comparative genomic hybridization analysis of *Apc*-mutant intestinal tumors allows the definition of minimal aneuploidy regions conserved between mouse and human and likely to encompass rate-limiting genes for intestinal tumor initiation and

progression. (*Am J Pathol* 2007, 170:377–387; DOI: 10.2353/ajpath.2007.060853)

Colorectal cancer (CRC) is caused by a multistep process that involves the accumulation of several genetic defects. Genetic instability facilitates the acquisition of these multiple gene hits, thus underlying colorectal tumor progression and malignant transformation. Chromosomal instability (CIN), in particular, is thought to cause aneuploidy as frequently observed in both sporadic and familial CRC.^{1,2}

Along the adenoma-carcinoma sequence characteristic of colorectal tumorigenesis, specific genetic alterations have been identified. Mutations in the adenomatous polyposis coli (*APC*) gene are considered as a rate-limiting step for adenoma formation both in familial (familial adenomatous polyposis; FAP) and sporadic CRCs. Mutations in the *KRAS* oncogene, found in ~50% of the cases, promote growth of the nascent adenomas. Loss of heterozygosity (LOH) at specific chromosomal regions, including 17p and 18q, characterizes more advanced stages and is thought to be centered around the *TP53* and *SMAD4* genes, respectively.

The progressive increase in genetic instability levels along the adenoma-carcinoma sequence in CRC is likely to be attributable to the acquisition of mutations in caretaker genes, such as *BUB1*, *BUBR1*, and *ATM*, with a broad spectrum of functional activities ranging from surveillance of chromosome segregation, response to DNA damage, cell cycle regulation, and mitotic checkpoint.

Supported by the Dutch Cancer Society (grant EMCR 2001-2482), the Dutch Research Council (grant NWO/Vice 016.036.636), and the Besluit Subsidies Investeren Kennisinfrastuctuur (BSIK) program of the Dutch Government (grant 03038).

Accepted for publication October 10, 2006.

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However, only a few of the above genes have been found to be mutated in CRC, and the incidence of these mutations is rather low.^{3–6} Notably, loss of *APC* function has been shown to result in structural mitotic defects (ie, microtubule attachment to the kinetochore and centrosomal abnormalities) and in both tetraploidy and aneuploidy, thus triggering CIN at the start of the adenoma-carcinoma sequence.^{7–10} Accordingly, low but significant levels of aneuploid changes have been observed at the adenoma stage both in sporadic and hereditary cases.^{2,11} Notwithstanding the latter, additional somatic hits seem to be necessary to promote the full-blown CIN phenotype observed in more advanced stages of CRC.¹

Mouse models carrying targeted *Apc* mutations provide unique tools for the analysis of *Apc*-driven CIN and for identification of those genes that synergize for CIN with the *Apc* tumor suppressor along the adenoma-carcinoma sequence.¹² However, in contrast to what has been observed in human intestinal tumors, very few somatic mutations, if any, have been reported to occur in these mouse intestinal tumors; in fact, no *Kras* or *Tp53* mutations have been found in gastrointestinal (GI) tumors from *Apc*^{+/-1638N} animals,¹³ and no other similar studies have been reported in the literature. Thus, to humanize the mouse model, we have bred the *Apc*^{+/-1638N} model^{14,15} with mice carrying transgenic and targeted mutations at the *KRAS* and *Tp53* genes to generate *Apc*^{+/-1638N}/*KRAS*^{V12G} and *Apc*^{+/-1638N}/*Tp53*^{-/-} compound mice. Here, we have used array-based comparative genomic hybridization (array CGH) to evaluate quantitative and qualitative aspects of CIN in intestinal tumors from the above mice when compared with those arising in the *Apc*^{+/-1638N} genetic background. Moreover, we have performed a cross-species comparison of the chromosomal regions more frequently affected by aneuploidy between adenomas from *Apc*-mutant mice and FAP patients carrying germline *APC* mutations.

Materials and Methods

Mouse Strains and Tumor Samples

The *Apc*^{+/-1638N} mice used in this study¹⁴ have been backcrossed to C57BL/6J for more than 20 generations and are regarded as fully inbred. *Apc*^{+/-1638N} animals were bred with the transgenic model pVillin-*KRAS*^{V12G} expressing the human *KRAS*^{V12G} oncogene under the control of the villin promoter.¹⁶ Because the latter model was available in the B6D2 (C57BL/6JxDBA/2) genetic background, the compound *Apc*^{+/-1638N}/*KRAS*^{V12G} animals used for the present study were backcrossed to C57BL/6J for five to seven generations to limit confounding effects by undesired genetic modifiers.

For the analysis of the effects of loss of *Tp53* function on *Apc*-driven aneuploidy, *Apc*^{+/-1638N} mice were bred with *Tp53*^{+/-tm1Tyj} (*Tp53*^{-/-}) animals carrying a constitutive deletion of the endogenous mouse *Tp53* gene.¹⁷ Compound heterozygous *Apc*^{+/-1638N}/*Tp53*^{+/-} mice were

then intercrossed to generate *Apc*^{+/-1638N}/*Tp53*^{-/-} experimental animals.

After macroscopic dissection of the GI, tumors of ~2 to 3 mm³ in size and normal tissues were snap-frozen in Tissue-Tec (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands) embedding medium in dry ice. Ten- μ m sections were briefly stained with hematoxylin and eosin (H&E), and consecutive sections were carefully laser-capture microdissected (LCM) using a PALM MicroBeam microscope system (P.A.L.M. Microlaser Technologies AG, Bernried, Germany) to limit contaminations from normal cells and obtain almost pure parenchymal cell samples. Adenomas were scored as either low or high dysplastic according to Boivin and colleagues.¹⁸ On average, 1500 parenchymal cells (900,000 μ m² area) were isolated from each tumor or normal specimen.

DNA Extraction and ϕ 29 Amplification

DNA extractions and ϕ 29 genomic amplifications were performed as previously described.¹⁹ All samples were quality-controlled by a polymerase chain reaction (PCR) to amplify for the mouse *Myh* gene with the following primers: mY5-2, 5'-CCTGGTGTCAAAGGCCTGA-3'; and mYe14, 5'-GCAGTAGACACAGCTGCAT-3'.

CGH Array, Labeling, and Hybridization

The mouse bacterial artificial chromosome (BAC) microarray slides here used for array CGH encompass 2803 unique BAC clones at 1 Mb spacing and were obtained from the Central Microarray Facility of The Netherlands Cancer Institute in Amsterdam.²⁰ DNA labeling was performed as previously described¹⁹ using a mixture of male genomic DNA extracted from the kidneys of two C57BL/6J mice as reference DNA. The Cy3-labeled sample and Cy5-labeled reference DNAs were precipitated together with 135 μ g of mouse Cot-1 DNA (Invitrogen, Breda, The Netherlands). DNA pellets were then redissolved in 120 μ l of hybridization buffer (50% formamide, 10% dextran sulfate, 0.1% Tween 20, 2 $\times$ standard saline citrate, and 10 mmol/L Tris-HCl, pH 7.4) together with 600 μ g of yeast tRNA (Invitrogen). Probe DNA was denatured for 10 minutes at 70°C and incubated for 1 hour at 37°C before application on the prehybridized BAC microarray slides. Array slides were prehybridized for 1 hour at 37°C with 90 μ l of denatured hybridization buffer containing 540 μ g of herring sperm DNA and 90 μ g of mouse Cot-1 DNA. Hybridizations were performed as previously described.²¹ After hybridization, slides were washed serially in solution 1 (0.05% Tween 20 in phosphate-buffered saline) for 10 minutes at room temperature, in solution 2 (50% formamide, 2 $\times$ standard saline citrate) at 42°C for 30 minutes, and twice in solution 1 at room temperature for 10 minutes. Finally, slides were spin-dried for 5 minutes at 1000 rpm. Image scans and their analysis were obtained by ScanArray Express HT (Perkin-Elmer Life Sciences, Boston, MA) and GenePix Pro 5.0 software (Axon Instruments, Union City, CA), respectively.

Data Analysis

BAC clone position map annotation and chromosomes order were according to Ensembl Build m34 (http://www.ensembl.org/mus_musculus/). All of the data were normalized using the Marray Tool and vsn packages into R environment as described elsewhere.² To facilitate detection of data trends and discriminate gain or loss events from variation introduced by the whole-genome amplification technique, we performed smoothing of the log2 ratio of the normalized data with the aCGH-Smooth software.²² Bioinformatic data analysis was performed using the Ensembl mouse genome server (http://www.ensembl.org/mus_musculus/) and the NCBI mouse genome resources (<http://www.ncbi.nlm.nih.gov/genome/guide/mouse/>). The analysis of mouse/human homology regions was performed by using the NCBI Comparative Maps, available at <http://www.ncbi.nlm.nih.gov/Homology/>.

Array CGH Data Validation by Single Nucleotide Polymorphism (SNP) Analysis

Selection of SNP markers polymorphic between the C57BL/6J and 129/Ola strains to be used as validation tools was performed by consulting the Mouse Genome Informatics (MGI) database (<http://www.informatics.jax.org/>). DNA extractions from LCM paraffin-embedded tumor sections from *Apc*^{+/-1638N} F1 129Ola/C57BL/6J mice was performed as previously described.²³ Two sets of primers were used to analyze SNPs rs3707129, rs3707206, and rs3707619 on chromosome 4 (Mb128.087890-953) and SNPs rs3704911, rs3704966, rs4136991, and rs4136994 on chromosome 5 (Mb107.544061-122), where two genomic regions were found to be frequently affected by gain/loss events. The primer sequences were as follows: chromosome 4 (forward) 5'-GCCTTCTGCTGTGTCTGAAG-3'; chromosome 4 (reverse) 5'-CCTTCTCTGAGGTTTGCTTGA; chromosome 5 (forward) 5'-GGGTGGCCAGACTGTTTAC-3'; and chromosome 5 (reverse) 5'-TTCCAAAGGTCCTGAGTTCAA-3'. PCR products were sequenced in both directions using the same primers. Sequencing was performed on an ABI 3700 capillary sequencer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Results

Genomic Profiling of *Apc*^{+/-1638N} Mouse Intestinal Tumors by Array CGH

To determine the presence of genomic alterations in mouse intestinal tumors driven by loss of *Apc* function, we performed array CGH analysis of LCM intestinal tumors derived from the *Apc*^{+/-1638N} mouse model.¹⁴ This method is highly sensitive and quantitative, and it allows the detection of chromosomal gains and losses in a small number of microdissected tumor cells with a resolution of ~1 Mb.^{2,19}

First, we analyzed 10 mouse intestinal tumors derived from *Apc*^{+/-1638N} animals encompassing both low ($n = 7$) and high ($n = 3$) dysplastic adenomas but no carcinoma.

Chromosomal aberrations were scored after smoothing the log2 ratio between normal and tumor DNA by aCGH-Smooth, a tool for automatic breakpoint identification and smoothing of array CGH data.²² To exclude putative artifacts attributable to the ϕ amplification procedure (see Materials and Methods), we excluded gain/loss events affecting single BACs. The results are depicted in Figure 1 as a heat-map overview of the chromosomal regions affected by aneuploidy throughout the tumor and normal samples analyzed in the study.

The four microdissected normal control samples collected from the intestine of inbred C57BL/6J wild-type animals displayed near-diploid genomic profiles with no aberrations in one case and up to two independent aberrations (an interstitial gain within chromosome 5 in two cases, and a loss within chromosome 11 in one case) in the remaining three cases. The same aneuploid changes were found in three (chromosome 5 gain) and two (chromosome 11 loss) tumors, respectively, and were accordingly excluded from all of the subsequent statistical analyses.

All *Apc*^{+/-1638N} tumor samples showed chromosomal number imbalances affecting either whole chromosomes or interstitial segments with a median of 11 events per sample (range, 4 to 21; SD = 6.5) (Table 1). No differences were observed in the number of aberrations between adenomas with high and low dysplasia (median, 12 and 10 events per sample, respectively). Notably, loss events were more frequent than gains among the tumor samples, with a median of two gain events per sample compared with 8.5 losses ($P < 0.001$, Wilcoxon signed ranks test). Gain of chromosome 1 (Mbp 94-130.6; 4 of 10 tumors) and losses of chromosome 2 (Mbp 25.1-33.7; 6 of 10 tumors), chromosome 4 (Mbp 117.4-153.1; 6 of 10 tumors), chromosome 5 (Mbp 108.5-148.5; 7 of 10 tumors), chromosome 8 (Mbp 121.7-125.9; 7 of 10 tumors), chromosome 10 (Mbp 80.1-80.6; 8 of 10 tumors), and chromosome 15 (Mbp 74.6-102.3; 9 of 10 tumors) were among the most frequently observed alterations (Figure 1). Whole chromosome loss/gain events were observed in three samples, namely loss of chromosome 8 (sample *Apc*9), loss of chromosomes 11 and 7 (sample *Apc*15), and loss of chromosomes 11 and 7 and gain of chromosome 18 (sample *Apc*18). The less frequent occurrence of gain/loss events at chromosome 18, where the *Apc* gene has been localized, confirms previous reports indicating that homologous somatic recombination is the principal pathway for allelic imbalance (AI) in adenomas in *Apc*^{+/-} mice, leading to duplication of the chromosome harboring the *Apc* mutant allele²⁴ (see Discussion).

Array CGH Validation by LOH Analysis Using SNPs

To validate the results obtained by array CGH on amplified LCM tumor samples, we performed LOH analysis by SNPs in an independent set of *Apc*^{+/-1638N} adenomas. Three sets of primers were designed to amplify the genomic regions on chromosomes 4 and 5 frequently lost in the *Apc*^{+/-1638N} mouse intestinal tumors. Each set of primers amplifies a region that contains at least three

Figure 1. Heat map visualization of the CGH results for the three groups of tumors analyzed in the present study. After normalization and two-log smoothing of the array CGH ratios from the 30 tumor samples and four normal intestinal epithelia, the data were loaded onto SpotFire Decision Site 8.1 to obtain the heat map here represented. Data are ordered in the X bar according to the sample genotypes as normal (N), *Apc*^{+/1638N} (*Apc*), *Apc*^{+/1638N}/*KRAS*^{V12G} (*Ras*), and *Apc*^{+/1638N}/*TP53*^{-/-} (*p53*), and in the Y bar according to the chromosome and Mb position of the BAC clones (color code at both sides of the heat map). The color code of the samples indicates BAC copy number changes: green, loss; red, gain; and black, no change.

SNPs known to be informative between the mouse strains C57BL/6J and Ola/129. To this aim, we used an independent group of tumors collected from *Apc*^{+/1638N} animals obtained by breeding C57BL/6J *Apc*^{+/1638N} mice with Ola/129 wild-type mice (*Apc*^{+/1638N} F1 C57BL/6J × Ola/129).

The presence of allelic imbalance was evaluated by direct sequence analysis of the PCR-amplified SNPs. LOH at chromosome 5 (Mbp 108.5-148.5) was observed

in four of seven tumors (57%) with concordant loss within the same PCR reaction of the Ola/129 alleles (Figure 2). Similar results were obtained with chromosome 4 (three of six, 50%) SNPs. The data not only confirm and validate the array CGH results relative to loss of distinct loci on chromosomes 4 and 5 but also indicate that allelic imbalance at these loci is a common event during *Apc*-driven intestinal tumorigenesis, acting independently of the genetic background.

Table 1. Aneuploid Changes Observed in Upper GI Polyps from $Apc^{+/1638N}$, $Apc^{+/1638N}/KRAS^{V12G}$, and $Apc^{+/1638N}/Tp53^{-/-}$ Mice

Genotype	Number of mice	Mean age (months)	Number of tumors analyzed	Medians of gains and losses/tumor (range)	Medians of gains and losses/tumor according to histology*
$Apc^{+/1638N}$	7	6	10	11.5 (4 to 21) ^{†‡}	1 (L); 10 (H)
$Apc^{+/1638N}/KRAS^{V12G}$	7	5	10	8.5 (2 to 25) ^{†§}	9.5 (L); 8.5 (H)
$Apc^{+/1638N}/Tp53^{-/-}$	4	3	10	9.5 (3 to 19) ^{‡§}	15 (L); 9 (H)

*Average number of chromosome alterations according to: H, high dysplastic adenomas; L, low dysplastic adenomas.

[†] $P = 0.513$; [‡] $P = 0.593$; [§] $P = 0.313$; Wilcoxon signed ranks test (two-tailed).

Genomic Profiles of Intestinal Tumors from $Apc^{+/1638N}/KRAS^{V12G}$ and $Apc^{+/1638N}/Tp53^{-/-}$ Animals

The above results indicate that aneuploidy occurs at very early stages of *Apc*-driven intestinal tumorigenesis, in agreement with our own array CGH analysis of human polyps from patients with germline *APC* mutations² but also with the previously reported *APC*'s function in mitosis and chromosomal stability.^{7,8,16} However, it is plausible that other genes frequently mutated along the adenoma-carcinoma sequence contribute, together with *APC*, to the full-blown CIN phenotype observed in late-stage CRC in human. To explore this hypothesis, we performed array CGH analysis of intestinal tumors from $Apc^{+/1638N}$ animals bred with either a transgenic model carrying the activated human *KRAS* oncogene under the control of the villin promoter ($KRAS^{V12G}$)¹⁶ or with a *Tp53* knockout model carrying a targeted *null* mutation at the endogenous *p53* tumor suppressor gene ($Tp53^{-/-}$).¹⁷ Tumors from these compound animals recapitulate the genetic

status of the vast majority of human colorectal tumors during adenoma progression ($Apc^{1638N}/KRAS^{V12G}$) and at later carcinoma stages ($Apc^{1638N}/Tp53^{-/-}$), and are therefore useful to test the above hypothesis. The phenotypic and molecular characterization of these compound models has been described elsewhere.^{15,25}

We performed array CGH analysis on 10 tumors from each of the compound $Apc^{+/1638N}/KRAS^{V12G}$ and $Apc^{+/1638N}/Tp53^{-/-}$ genotypes, encompassing both low and high dysplastic adenomas (two low and eight high in the former, and three low and seven high in the latter). The results of these analyses are reported in Figure 1 and Table 1. Intestinal adenomas from $Apc^{+/1638N}/KRAS^{V12G}$ animals showed a median of chromosomal alterations of 8.5 events per sample (range, 2 to 25; SD = 6.8). The latter does not significantly differ from the average number of chromosomal alterations found in $Apc^{+/1638N}$ tumors (median, 11 events per sample; range, 4 to 21; SD = 6.5). Moreover, no statistically significant differences were observed between low and high dysplastic lesions, although the sample size is admittedly limited (Table 1). Tumors from $Apc^{+/1638N}/Tp53^{-/-}$ mice presented a median of 9.5 gain/loss events per sample (range, 3 to 19; SD = 5.08) with low dysplastic adenoma characterized by a slight though not significant increase in the number of genomic imbalances when compared with the high dysplastic group (median, 15 versus 9). Moreover, no significant differences were found between the three groups when loss and gain events were analyzed separately. However, when gains and losses affecting whole chromosomes were considered, tumors from $Apc^{+/1638N}/Tp53^{-/-}$ mice revealed a significant increase when compared with intestinal lesions from $Apc^{+/1638N}$ (median of one event per sample compared with 0; $P = 0.008$; Wilcoxon signed ranks test) or $Apc^{+/1638N}/KRAS^{V12G}$ (median of one event per sample compared with 0; $P = 0.005$; Wilcoxon signed ranks test). In addition, when the total number of gains versus losses for all three tumor genotypes is taken into consideration, loss events seem to occur at higher frequency regardless of genotype (median of eight versus two; $P < 0.001$; Wilcoxon signed ranks test).

Overall, the array CGH analysis performed on intestinal tumors from $Apc^{+/1638N}$, $Apc^{+/1638N}/KRAS^{V12G}$, and $Apc^{+/1638N}/Tp53^{-/-}$ mice led to the identification of several chromosomal abnormalities, with evidence of intertumor heterogeneity even among tumors from within the same genotype (Figure 1). However, we also observed the presence of recurrent genomic aberrations likely to result from selection of specific chromosomal regions encompassing

Figure 2. LOH analysis performed on $Apc^{+/1638N}$ F1 C57BL/6J $\times$ Ola/129 mouse tumors by direct nucleotide sequencing of single nucleotide polymorphisms (SNPs) validates the array CGH results. The examples are relative to three SNPs on chromosome 5: rs3704966, rs4136991, and rs4136994. **A:** Nucleotide sequence analysis of SNP rs3704966 showing retention of both alleles in the tumor sample. **B** and **C:** Nucleotide sequence analysis of SNPs rs3704966 or rs4136991 and rs4136994, respectively, each showing hetero- and hemizygosity in the normal and tumor DNA samples, respectively. The arrows indicate the polymorphic nucleotide position for each SNP sequence.

Figure 3. Overview of the chromosome copy number changes throughout the 30 mouse intestinal tumors analyzed in this study. Total numbers of all significant gains or loss regions found to be affected by copy number alterations in more than or equal to two tumor samples from at least two independent groups have been plotted separately according to the tumor genotype. Complete or partial chromosome gains and losses are shown separately. Regions are ordered according to their mapped positions along the chromosomes. G, gain; L, loss of the corresponding regions.

highly relevant genes for intestinal tumor progression. All of the chromosomal segments found to be affected by copy number alterations in at least two independent tumor samples from two independent groups have been plotted and are depicted in Figure 3. Specific chromosomal regions of recurrent aneuploidy are present throughout all three adenoma groups; in particular, chromosomes 2, 4, 5, 8, 10, and 15 show copy number alterations in more than 60% of the samples in at least two distinct genotypes. Notably, a total of six (20%) allelic imbalance events (gains and losses) were found at chromosome 18, where the *Apc* and *Smad4* tumor suppressors are localized.

Comparative Genomic Analysis of Mouse and Human Intestinal Adenomas Reveals Highly Conserved Regions of Aneuploidy

To identify chromosomal loci conserved between human and mouse that recurrently undergo gain/loss events during intestinal tumor progression, we performed a comparative analysis of the mouse array CGH profiles with those derived from our previous array CGH analysis of early adenomas from FAP patients carrying established *APC* mutations.² To this aim, we used the human-mouse comparative maps available through the National Center of Biotechnology Information, National Institutes of Health (<http://www.ncbi.nlm.nih.gov/Homology/>). Based on the analysis of the homologous segments, the vast majority of the numerical chromosomal changes found in the mouse correspond to chromosomal regions of frequent allelic imbalance among

FAP adenomas (Table 2). Notably, chromosomal regions with the highest deletion frequencies among mouse intestinal tumors are syntenic to human chromosomal regions with the highest LOH incidence in FAP polyps. Mouse chromosomes 4 and 15 for instance, harbor several regions that are orthologous to human chromosome 1p34-p36 and 22q12-q13, respectively, both known to be lost in human FAP adenomas with a percentage that varies between 67 to 75%. In some cases, the analysis of the homology region between mouse and human chromosomal regions of allelic imbalance allows the definition of a minimal common region, thus providing a powerful gene-discovery tool (see Discussion).

Discussion

Extensive genome-wide chromosomal alterations, indicative of CIN, characterize the vast majority of human CRCs.^{26,27} However, the molecular basis of CIN in CRC is still poorly understood, and although a number of genes have been characterized that may underlie aneuploidy (eg, *BUB1*, *BUBR1*, and *CDC4*),^{5,28,29} the frequency at which mutations have been found in their coding regions is too low to justify the high CIN incidence found in human CRC.²⁹ Notably, loss of *APC* function results in microtubule plus-end attachment defects during mitosis and consequent chromosome misalignment and CIN.⁷⁻⁹ These observations, made in cultured mammalian cells, indicate a potential role for the *APC* tumor suppressor gene in triggering CIN right from the start of

the adenoma-carcinoma sequence. Accordingly, our array CGH analysis of early-stage adenomas from FAP patients carrying germline *APC* mutations² and from *Apc*-mutant mouse models (this study) confirm that aneuploidy is indeed found in nascent benign tumors.

It is generally accepted that the main tumor-suppressing activity of the *APC* gene resides in its capacity to regulate intracellular β -catenin levels as part of the canonical Wnt signal transduction pathway.¹ Hence, the observation that aneuploidy occurs at very early stages of the adenoma-carcinoma sequence is in agreement with a model in which the initial loss of *APC* function triggers intestinal tumor formation by constitutive activation of Wnt/ β -catenin signaling and simultaneously results in a low but significant level of CIN. At later stages of tumor progression, somatic mutations in genes involved in mitotic and cell cycle checkpoints, telomere shortening and telomerase expression, centrosome number regulation, and double-strand break repair may work synergistically with the kinetochore and chromosome segregation defects caused by *APC* mutation in eliciting full-blown CIN.^{1,10}

Overall, our array CGH analysis has revealed chromosomal aberrations in each of the 30 mouse GI polyps analyzed. Even in the case of small low dysplastic *Apc*^{+/-1638N} adenomas (2 mm³), an average of 12 events (gains and losses) per tumor sample is found. Notably, both in *Apc*^{+/-1638N} as well as in FAP adenomas, loss events were more frequently observed than gains.² This relatively high incidence of aneuploid changes at very early stages of the adenoma-carcinoma sequence appears to be in contrast with previous reports in which intestinal adenomas from *Apc*^{+/-Min} mice³⁰ and from FAP patients with known *APC* mutations³¹ were shown to have stable karyotypes. This apparent discrepancy is likely to result from the different methods used in these studies. Conventional karyotype analysis and fluorescence-activated cell sorting are less sensitive than array CGH in detecting more subtle subchromosomal changes leading to allelic imbalance. Accordingly, by using a more sensitive PCR-based method, Shih and colleagues¹¹ also reported widespread allelic imbalance in human sporadic adenomas. As shown in the present and previous study,² the quantitative analysis of genomic changes by coupled LCM and whole genome BAC arrays, allows the detection of gain/loss events in small intestinal lesions (1 to 2 mm²) with relatively high sensitivity and without the noise introduced by contaminating normal lymphocytes and stromal cells.

Although our results do not allow us to speculate on the mechanisms underlying this early genomic instability, we have established that aneuploid changes are observed at early stages of *APC/Apc*-driven intestinal tumorigenesis in both human² and mouse (this study). The question remains whether this genomic instability precedes the rate-limiting second hit at the *Apc* gene or it arises as a consequence of it. In the mouse genome, the *Apc* gene maps to chromosome 18. In agreement with the Knudson's two hit model, the vast majority of intestinal tumors from *Apc*-mutant mice show LOH at the chromosome 18 region encompassing the wild-type *Apc* allele.^{13,32,33} As elegantly shown by Haigis and Dove,²⁴ somatic mitotic recombination (and not aneu-

ploidy) is the principal mechanism underlying loss of the wild-type *Apc* allele in the *Apc*^{+/-Min} mouse model. Because mitotic recombination does not affect copy number, it cannot be detected by array CGH analysis. Accordingly, our array CGH analysis revealed aneuploidy at chromosome 18 only in a minority of adenomas from *Apc*^{+/-1638N} (10%) and from *Apc*^{+/-1638N}/*KRAS*^{V12G} and *Apc*^{+/-1638N}/*Tp53*^{-/-} (25%) animals. Hence, our results indirectly confirm that, in the majority of the cases, the rate-limiting LOH event at the *Apc* locus does not occur by interstitial deletion or nondisjunction, and is likely to result from mitotic recombination as shown for *Apc*^{+/-Min}. However, aneuploid changes at the *Apc* locus on chromosome 18 still take place in a fraction of the intestinal adenomas initiated by the *Apc*^{1638N} targeted mutation, possibly as the result of low but significant CIN levels. That *APC* mutations may act in a dominant-negative manner in eliciting mitotic defects^{9,34} may underlie the chromosome 18 aneuploid changes observed by array CGH in adenomas from *Apc*^{+/-1638N}. In these cases, genomic instability may even precede the rate-limiting second hit at the wild-type *Apc* allele. Nevertheless, in the majority of the cases, more significant CIN levels will occur only on complete loss of *Apc* function, ie, after the somatic hit.

During the adenoma-carcinoma progression in CRC, genetic instability progressively increases with the accumulation of somatic mutations at specific tumor suppressor genes and oncogenes.³⁵ The occurrence of *KRAS* mutations already at early adenoma stages³⁶⁻³⁸ suggests the possibility that this oncogene may partly contribute to CIN. In fact, few studies have reported positive correlations between *KRAS* mutations and aneuploidization.^{39,40} It has been postulated that constitutive *KRAS* activation may induce genomic instability via the mitogen-activated protein kinase (MAPK) pathway, which affects G₁ and G₂/M cell-cycle transit times and apoptosis.⁴¹ Moreover, *KRAS* can also have a role in cytoskeletal and microtubule organization of the mitotic spindle, through the regulation of Rho-like GTPases.⁴² Notwithstanding these data, our results do not demonstrate any increase in the incidence and type of allelic imbalance in tumors from compound *Apc*^{+/-1638N}/*KRAS*^{V12G} mice when compared with *Apc*^{+/-1638N} animals. These results are also corroborated by other studies on human CRC: no association was found between the mutational status of the *KRAS* gene and the number of chromosomal aberrations⁴³ or genomic instability.⁴⁴

The case for loss of p53 function and CIN is a more solid one. First, the p53 tumor suppressor protein has a well-characterized role as DNA damage checkpoint.⁴⁵ Moreover, several cancers characterized by aneuploidy harbor p53 mutations both in human⁴⁶⁻⁴⁸ and in mouse models.⁴⁹ Loss of p53 function is also known to result in an increase in tetraploid and polyploid cells.^{50,51} Although the CGH array technique used here does not allow to detect tetraploidy, our results indicate a significant increase in whole chromosome gains and losses among *Apc*^{+/-1638N}/*Tp53*^{-/-} adenomas when compared with tumors from *Apc*^{+/-1638N} and *Apc*^{+/-1638N}/*KRAS*^{V12G} mice. It has been shown that p53-deficient cells fail to arrest in G₁ leading to tetraploidization and, on further progression through the cell cycle, to abnormal mitoses

Table 2. Analysis of the Mouse-Human Chromosome Synteny and Incidence of Genomic Aberrations in Intestinal FAP Adenoma and Sporadic CRC

Mouse chromosome segment (Mbp)	Incidence in mouse adenomas	Human syntenic region (cytogenetic band)	Incidence in human FAP ²		Incidence in human CRC ^{68-73*}	
			As gain	As loss	As gain	As loss
Gains						
1 (94 to 130)	33%	5q21 18q21q22-q23 2q12-q13-q14 2cen-q13 2q21-q22		5q21 (13%) 18q (4%)		5q (16%) 18q (42%) 2q (6%)
4 (117 to 153)	6%	1p34-p36		1p36 (57%) 1p (75%)		1p (17%)
8 (whole chr.)	6%	13q33-q34-q14 8p21-p22-p23-p11 4q31-q32-q33-q34-q35-21-q28 19p13-p12 22q13 16q11-q12-q13 16q21-q22-q23-q24 1q42 10p11	1q42 (8%) 10p (2%)	13q (4%) 8p (2%) 19p (73%) 22q13 (63%) 16q (8%) 1q42 (4%)	22q13 (5%) 16q (7%)	8p21p22 (28%) 4q31-q34 (21%)
11 (whole chr.)	6%	22q12 17q25 7p12-q13	7p (8%)	22q (67%) 17q25 (33%)		22q (6%)
13 (0 to 40)	12%	10p14-p15 1q41-q42-q43 7p14-p15 6p21-p22-p24-p14	7p (19%)	10p (2%) 1q (4%)	7p (35%) 6p21 (7%)	
18 (13 to 23)	12%	18q11-q13		18q (4%)		18q (42%)
18 (whole chr.)	6%	10p11 18q11-q12 2q13-q13-q21 5q21-q22-q23-q31-q32-q33 18p11-q21-q22-q23	18q (2%)	10p (2%) 18q (4%) 5q (12%) 18q21 (4%)		18q (42%) 5q (16%) 18p (26%) 18q21 (60%)
Losses						
2 (25 to 33)	50%	9q34		9q (36.5%)	9q (8%)	
2 (152 to 181)	30%	20q11-q13		20q (36.5%)	20q13 (50%)	
4 (117 to 153)	56%	1p34-p36		1p36 (57%) 1p (75%)		1p (17%)
5 (114 to 148)	66%	12q24 13q12 7p11-p22-q11-q22	7p (19%) 7q (19%)	12q (50%) 13q (7.7%)	13q (12%)	
7 (0 to 23)	16%	19q13		19q13 (68%)		
7 (whole chr.)	16%	19q13 11p15-p14 15q11 2p13 15q26 11q13-q14-p15 16p11-p12-p13 1-q26		19q13 (68%) 15q (4%) 16p (2%) 16q (8%)	11q (43%) 16p13 (5%)	
8 (121 to 125)	63%	16q24 1q42	1q (8%)	16q (8%) 1q (4%)		
10 (80.1 to 80.6)	86%	19p13.3		19p (73%)		
11 (whole chr.)	20%	22q12 17q25 7p12-q13	7p (8%)	22q (67%) 17q25 (33%)	7p (25%)	22q (6%)
11 (117 to 118)	60%	17q25		17q (42%)		17q25 (14%)
13 (whole chr.)	10%	10p15 1q41-q42-q43 7p14-p15 6p21-p22-p23-p24-p25 9q21-q22 5q34-q35-q31 9q21-q22 8q22 5p15 5q11-q12-q13- q14-q15	7p (19%) 8q (2%)	10p (2%) 1q (4%) 5q (14%) 5q (11%) 8q (15%)	7p (35%) 6p21 (7%) 9q21 (6%) 8q (63%) 5p (4%)	5q (16%)
13 (48 to 69)	10%	5q34-q35-q31 9q21-q22 8q22 5p15		5q (14%) 8q (15%)	9q21 (6%) 8q (63%) 5p (4%)	5q (16%)
15 (74 to 102)	70%	22q12-q13 12q12-q13		22q (67%) 12q (50%)	12q (5%)	12q (15%)
17 (whole chr.)	6%	6q25-q27 5q15 19q13 6p21 21q22 10p13 7p21-p22 3p24 19p13 5q21 18p11 2p21-p22-p23-p16 18p11	7p (20%)	19p (73%) 5q (12%) 19q13 (58%) 21q (10%)	5q (16%) 7p (25%)	5q (16%) 7p (25%)
17 (21 to 32)	20%	19p13		19p (73%)		

(table continues)

Table 2. Continued

Mouse chromosome segment (Mbp)	Incidence in mouse adenomas	Human syntenic region (cytogenetic band)	Incidence in human FAP ²		Incidence in human CRC ^{68-73*}	
			As gain	As loss	As gain	As loss
18 (whole chr.)	6%	10p11 18q11-q12 2q13-q13-q21 5q21-q22-q23- q31-q32-q33 18p11-q21-q22- q23		10p (2%) 18q (4%) 5q (12%) 18q21 (4%)		18q (42%) 5q (16%) 18p (26%) 18q21 (60%)
18 (30 to 93)	13%	18q11-q12 2q13- q13-q21 5q21- q22-q23-q31-q32- q33 18p11-q21- q22-q23		18q (4%) 5q (12%) 18q21 4%)		18q (42%) 5q (16%) 18p (26%) 18q21 (60%)
19 (0 to 9.4)	53%	11q11-q12-q13			11q (19%) 11q13 (43%)	

*Results are described in References 68 to 72 and are summarized in Reference 73.

and aneuploidy.⁵² This mechanism seems to be corroborated by the increased incidence of whole chromosome loss and gain in p53-deficient intestinal tumors.

The array CGH analysis of mouse intestinal adenomas revealed a number of chromosomal loci where allelic imbalances recurrently occur in independent tumors and throughout the three genotypes. It is therefore likely that mutations in genes encompassed within these chromosomal regions are selected at early developmental stages of intestinal tumor onset and progression. However, the chromosomal intervals affected by these recurrent aneuploidy events are usually very large, which makes the identification of individual genes underlying tumor onset and progression a formidable task. Throughout the mouse intestinal adenomas analyzed here, loss of chromosome 10 (Mb 80.1 to 80.6) and of the distal region of chromosome 15 (Mb 74.6 to 102.3) were the most frequent alterations with frequencies of 86 and 70%, respectively. The limited size (~500 kb) delineated by the loss events within chromosome 10 somewhat facilitates the search for putative candidate cancer-related mapping within the Mb 80.1 to 80.6 interval. *In silico* analysis of the mouse genome sequence (<http://www.informatics.jax.org/>) revealed 40 annotated expressed sequences, 26 of which are known genes. The localization of the *Stk11* gene within this region is of particular interest in view of the causative role of this serine/threonine kinase in Peutz-Jeghers syndrome, an inherited susceptibility to intestinal cancer characterized by multiple hamartomatous polyps throughout the upper GI tract and a high risk of developing cancer in the intestine and other organs.⁵³

To further restrict the size of the chromosomal intervals affected by gain/loss events and pinpoint specific conserved genes that play rate-limiting roles in intestinal adenoma formation in the mouse and human, we compared array CGH profiles from early FAP adenomas (obtained from carriers of known *APC* germline mutations) and from histology-matched intestinal tumors from *Apc*^{+/-1638N} mice. The results of this comparative analysis (Table 2) have indicated the presence of a surprisingly high number of chromosomal aberrations shared between mouse upper GI and human colorectal

adenomas, notwithstanding the different anatomical location of these two groups of GI tumors. The above-mentioned mouse chromosome 10 region (Mb 80.1 to 80.6), where aneuploid changes have been observed in 86% of the mouse adenomas here analyzed, is orthologous to human chromosome 19p13.3, where LOH has been reported in 73% of FAP adenomas. Besides *STK11*, also the *APC2* gene,^{54,55} previously proposed as potential tumor suppressor gene in ovarian and lung cancer,^{56,57} map to the 19p13.3 chromosomal interval.

Another example is provided by the interval on mouse chromosome 15 (Mb 74 to 102), the second most frequent (70%) site of allelic imbalance in tumors from *Apc*^{+/-1638N} mice. This region is syntenic to human chromosomes 22q12-q13 and 12q12-q13, both previously reported to undergo LOH in FAP adenomas at high frequencies (67 and 50%, respectively).² Notably, at least three genes with known tumor suppressor functions in the GI tract map in human to chromosome 22q13. *ST13* (suppression of tumorigenicity 13) maps to chromosome 22q13.2 and was reported to be down-regulated in CRC tissue when compared with its expression in adjacent normal tissue.⁵⁸ Bi-allelic mutations of p300, a transcriptional co-activator binding E1A also localized to chromosome 22q13.2, have been shown in several human cancers. In particular, this gene seems to function as a tumor suppressor gene in the intestinal epithelium,⁵⁹ and mutations in its coding region are found at high frequency among CRC cell lines.⁶⁰ *NBK/BIK* is another putative tumor suppressor gene localized within a 0.5-cM region of chromosome 22q13 known as the target of frequent allelic loss in human CRC.⁶¹ The proapoptotic function of this *BCL-2* family member indeed suggests a role in tumor suppression for this protein.⁶²

The frequently lost interval on mouse chromosome 4 (Mb 117 to 153) is orthologous to human chromosome 1p34-p36. Among FAP adenomas, chromosome 1p36 shows loss in ~60% of the cases,² thus restricting the candidate tumor suppressor genes in this 24-Mb region, to a total of 392 transcripts. Among these, the meningioma suppressor gene *ALPL*,⁶³ the prostate cancer susceptibility gene *HSPG2*,⁶⁴ and *CASP9*, a proapoptotic

component of the caspase cascade,⁶⁵ represent interesting and worth investigating candidates.

In addition, the mouse chromosome 2 region (Mb 25 to 33) lost in 50% of the *Apc*^{1638N} adenomas, is syntenic with human chromosome 9q34. Again, chromosome 9q is frequently lost among FAP polyps.² The additional information obtained from the analysis of mouse intestinal tumors allows restriction of the search for candidate tumor suppressor genes to the chromosome 9q34 interval where cancer-related genes such as *TSC1* (tuberous sclerosis 1)⁶⁶ and *DAPK1* (death-associated protein kinase 1),⁶⁷ are known to be localized. The use of additional comparative tools, such as expression profiling analysis, is likely to further pinpoint critical genes among the still large number of transcripts ($n = 227$) mapping to this subchromosomal region.

In conclusion, we have shown that allelic imbalance and aneuploid changes occur at early stages of *Apc*-driven intestinal tumorigenesis in mouse. Whereas introduction of an oncogenic *KRAS* mutation does not alter the overall frequency and type of aneuploid changes, loss of *Tp53* function increases the incidence of whole chromosome loss and gain events. Notably, the chromosomal intervals frequently affected by aneuploid changes in *Apc*-mutant tumors are syntenic to genomic regions known to be frequently lost or gained in adenomatous polyps from FAP carriers of germline *APC* mutations. This comparative genomic profiling approach, when combined with gene expression signatures from human and mouse tumors and coupled with functional tumor suppression assays, will allow the identification of novel genes involved in intestinal tumor onset and progression.

Acknowledgment

We thank Dr. Claudia Gaspar for technical assistance and stimulating discussions.

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