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Loss of Heterozygosity and Nonsense Mutation in *Apc* in Azoxymethane-induced Colonic Tumours in *Min* Mice

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Abstract. *C57BL/6J Min/+ mice, which carry a nonsense mutation in Apc, were injected twice neonatally with 5 mg azoxymethane (AOM) /kg body weight. AOM treatment in comparison with untreated Min mice increased the incidence and number of colonic tumours from 6/14 to 22/24 (incidence) and 0.64±0.9 to 4.0±3.5 tumours per mice, respectively. Colonic tumours were analysed for loss of heterozygosity (LOH) in Apc, and 32 of the samples showed LOH whereas 14 did not. In untreated Min mice, all 8 tumours had LOH in Apc. All tumour samples from the AOM-treated Min mice were analysed for nonsense mutations between codons 686 and 1217 in the Apc gene, and one sample had a G→A transition mutation in codon 1047. No β-catenin mutations in the region coding for phosphorylation sites important for degradation were found. In conclusion, the main mechanism for colonic tumour induction in AOM-induced Min mice is LOH in Apc, but Apc nonsense mutations may also occur.*

C57BL/6J multiple intestinal neoplasia (*Min*) mice, which spontaneously develop intestinal adenomas, carry a heterozygous germline mutation in the *Apc* gene that changes codon 850 in *Apc* to a stop codon (1, 2). Apc is a multifunctional protein that controls the degradation and thereby intracellular levels of the oncogene β-catenin (3). In turn, β-catenin regulates genes that have important roles in the development and progression of colorectal carcinoma (4). The *Min* mouse is a murine model of the human syndrome familial adenomatous polyposis, which is also caused by germline mutations in the *APC* gene (1). Intestinal tumour formation in *Min* mice is predominantly associated with loss of the wild-type *Apc* allele (5), but also truncating mutations, particularly following exposure to a carcinogen (6).

We and others have previously shown that AOM treatment of *Min* mice increases tumour development, particularly in the colon (7, 8). The most likely initiating event in carcinogenesis by methylating agents is methylation of O^6 in the DNA base guanine (9). Presence of unrepaired O^6-methylguanine causes G→A transition mutations following two rounds of replication (10). Whereas AOM and its precursor 1,2-dimethylhydrazine induce intestinal tumourigenesis in rats and mice by causing G→A and C→T mutations in codons of β-catenin important for its degradation (11-14), this does not seem to occur in *Min* mice, where no β-catenin mutations in intestinal tumours were observed (8). In our previous study, however, we showed that AOM-treated *Min* mice had both colonic tumours and precursor lesions ACF_{Min} with overexpression of β-catenin (15). This accumulation of β-catenin in the absence of β-catenin mutations (8) could be explained by loss of Apc function, either *via* loss of the wild-type *Apc* allele or mutations. Absence of wild-type Apc, as shown by immunohistochemistry in a small sample of colonic tumours of AOM-treated mice (16), would support this hypothesis. In this study, we therefore further investigated genetic changes in colonic tumours of AOM-induced *Min* mice by analysing for loss of heterozygosity (LOH) in the *Apc* gene and searched for mutations in the *Apc*, as well as β-catenin genes.

Materials and Methods

Mice breeding. C57BL/6J *Min*/+ mice were bred at the Norwegian Institute of Public Health, Oslo, Norway, from mice originally purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Procedures to secure inbreeding (<12 generations) were followed. C57BL/6J-*Min*/+ males and C57BL/6J-+/+ (wild-type) females (Bomholt, Denmark) were mated and the *Min*/+ offspring were identified by an allele-specific PCR-assay as described previously (17). The mice were fed a breeding diet, SDS RM3 (E), from Special Diets Services Ltd. (Witham, UK) during gestation and until 5 weeks of age; thereafter they were given a standard maintenance diet from B&K Universal Ltd (Grimston, UK). Water and diet were given *ad libitum*.

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AOM treatment. AOM (Sigma Chemical Co., St Louis, MO, USA) was diluted in 0.9% NaCl and 5 mg/kg was injected (10 μl/g body weight) *s.c.* at 1 and 2 weeks after birth.

The mice were killed by CO_2 gas and cervical dislocation at 11 weeks of age or older. The abdomen was cut open, the colon was removed and flushed with ice-cold PBS (1.14 mM $NaH_2PO_4 \bullet H_2O$, 5.53 mM $Na_2HPO_4 \bullet 12H_2O$, 0.14 M NaCl, pH 7.4). The colon was cut open longitudinally, washed and fixed flat between filter paper 2 (Whatman International Ltd, Maidstone, England) for 24 h in absolute ethanol (AS Vinmonopolet, Oslo, Norway). The tissues were examined by transillumination in a light microscope (Nikon TMS-F, Melville, NY, USA) in order to score tumours.

Tumour samples. In this study 78 colonic tumour samples were collected from 11 male and 13 female *Min* mice treated with AOM, and 9 colonic tumour samples from 10 male and 4 female control *Min* mice not treated with AOM.

Colonic tumour samples were collected from the centre of the tumours by puncture with a cannula (diameter 0.5 mm) and treated with 60 μl 100 units/ml collagenase (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) at 37˚C for 20 min and 6.72 μl trypsin EDTA (Bio Whittaker Europe, Verviers, Belgium) at 37˚C for 12 min before grinding. DNA isolation was performed as described previously (18).

Detection of LOH. In order to assess the *Apc* allele status, LOH analysis (18) was performed on all samples at least three times. The principle is that the wild-type Apc$^+$ allele contains a *Hind*III restriction enzyme site that is not present in the Apc^{Min} allele. After a PCR reaction (5) followed by enzymatic cutting and allelic separation, the ratio between the Apc$^+$ and mutated allele were determined quantitatively by the Gel-Pro Analyzer version 3.0 (Media Cybernetics, MD, USA).

Analysis of Apc truncation mutation. Nonsense mutations in the *Apc* gene were analysed by a slightly modified *in vitro* synthesised protein (IVSP) assay (19, 20). The IVSP assay covered segment 2 (codons 686-1217) in exon 15 of the *Apc* gene and was analysed as described (5, 18). All samples were analysed by the IVSP assay once, and samples that did not show LOH were analysed a second time to confirm the previous analysis.

To verify the truncation mutation, isolated DNA from nucleotides 1974 to 3782 of the Apc gene (including segment 2 from the IVSP assay) was amplified as described (6, 21).

The PCR product was cloned into the pCR2.1-TOPO vector (Invitrogen, Groningen, The Netherlands) according to the manufacturer's protocol for the TOPO TA Cloning kit (Invitrogen). Thirty-nine colonies were picked and grown overnight in LB medium at 37˚C before plasmid isolation was performed according to the Wizard® SV 96 Plasmid DNA Purification System (Promega Corp, Madison, WI, USA). Isolated plasmid DNA was double-digested with BamHI and EcoRV (New England Biolabs, Beverly, MA, USA) to verify the correct length of the insert. The colonies were further screened for Apc^+ and Apc^{Min} alleles as previously described (17, 22, 23). Thirteen colonies contained the Apc^+ allele and were selected for IVSP analysis to confirm the truncation mutation prior to sequencing.

Sequencing of plasmid DNA containing the *Apc* truncation mutation was performed according to the CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckmann Coulter, Inc., Fullerton, CA, USA). Briefly, DNA was measured according to the PicoGreen® dsDNA Quantitation Reagent and Kits and read using a VersaFlour™ Flourometer System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The template was diluted in distilled water and pre-heated to 95˚C for 20 min. The sequencing reaction contained diluted template, 1 μl primer (1.6 μM) and 4 μl DTCS Quick Start Master Mix in a total reaction of 10 μl. Primers used for sequencing were selected after estimation of the location of the truncation mutation in the IVSP assay. The template was sequenced in both directions, with the forward primer 5'-CCC AGA TAG CCA AAG TTA TG-3' and reverse primer 5'-ACC TAT ATG GGG AAA CAC ATT-3' (21). The cycling conditions were 95˚C for 2 min, followed by 35 cycles with 96˚C for 20 sec, 50˚C for 20 sec and 60˚C for 4 min. Unincorporated dye terminators were removed from the sequencing reaction according to the DyeEx 2.0 Spin Kit (Qiagen GmbH, Hilden, Germany). Clean and dry samples were dissolved in 20 μl Sample Loading Solution (Beckman Coulter Inc.), overlaid with mineral oil, run and analysed by the CEQ 8000 Genetic Analysis System (Beckman Coulter Inc.).

Sequencing of β-catenin. Tumour samples that did not show LOH were analysed for β-catenin mutations in a 150-bp segment corresponding to functionally important phosphorylation sites (24). Isolated tumour DNA was amplified with sense 5'-GGA GTT GGA CAT GGC CAT GG-3' and antisense 5'-TCC ACA TCC TCT TCC TCA GG-3' primers in a 30 μl mixture containing 1x PCR buffer II, 200 μM dNTP's, 0.5 μM of each primer, 1 mM $MgCl_2$, and 0.3 μl of AmpliTaq Gold DNA Polymerase (PE Applied Biosystems, CA, USA). Cycling conditions were 94˚C for 10 min, followed by 37 cycles (94˚C for 1 min, 61˚C for 2 min, 72˚C for 3 min) with a final extension at 72˚C for 7 min. For purification of the PCR products, 5 μl PCR product and 2 μl ExoSAP-IT™ (Amersham Biosciences, AB, Uppsala, Sweden) were incubated at 37˚C for 15 min, followed by 80˚C for 15 min to inactivate the enzymes. The purified PCR products were sequenced according to the CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter Inc.). In short, purified PCR products were diluted and heated to 95˚C for 1 min. Denatured DNA was mixed with 4 μl DTCS Quick Start Master Mix and 1 μl primer (1.6 μM) in a total volume of 10 μl. The DNA was sequenced in both directions by using the same oligonucleotides as in the PCR reaction. Cycling conditions were 94˚C for 2 min, followed by 30 cycles with 96˚C for 27 sec, 50˚C for 27 sec and 60˚C for 4 min. Unincorporated dye terminators were removed as described above and analysed by the CEQ 8000 Genetic Analysis System (Beckman Coulter Inc.).

Table I. *Incidence, number and LOH in Apc of colonic tumours in Min mice.*

Treatment	Incidence	Mean no. of tumours±SD	LOH	Not-LOH
AOM	22/24[a]	4.00[b]±3.49	32/46[c]	14/46
Control	6/14	0.64±0.93	8/8	0/8

[a] $p=0.002$, Fisher Exact Test, incidence different from control.
[b] $p<0.001$, One-way Anova on ranks, number of tumours different from control.
[c] $p=0.095$, Chi-square, proportion not significantly different from control.

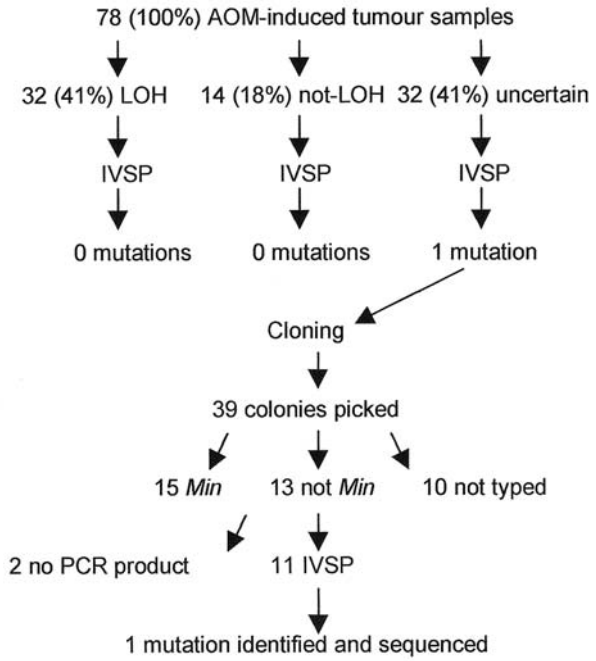

Figure 1. *Experimental outline of screening of colonic tumours from AOM-treated Min mice for nonsense mutation analysis in segment 2 (codons 686-1217) in exon 15 of the Apc gene. See Materials and Methods for further details.*

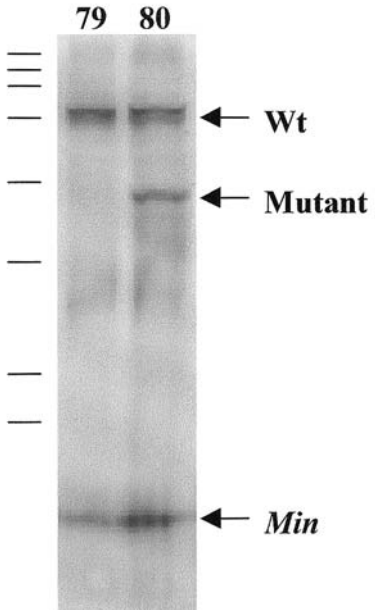

Figure 2. *IVSP assay performed on DNA isolated from colonic tumours of AOM-treated Min/+ mice. Segment 2 (codons 686-1217) in exon 15 of the Apc gene was amplified and the PCR products used in a coupled in vitro transcription-translation reaction with [^{35}S]methionine. The polypeptides were separated on 15% Tris-HCl PAGE Ready gel and the films were exposed to radiography. Sample 79 shows the wild-type and the Min band, whereas sample 80 in addition has a mutant band of about 45 kD. Molecular weight markers are indicated on the left, starting from the top: 250, 160, 105, 75, 50, 35, 30 and 25 kD.*

Statistical analysis. For comparison of tumour number from the treatment group, we used one-way ANOVA on ranks for non-parametric data, followed by the appropriate multiple comparison procedure (SigmaStat software, Jandel Scientific, Erkrath, Germany). Fisher's exact probability test was used to evaluate incidence data. For analyses of differences in the frequency of LOH, we used Chi-square analyses.

Results and Discussion

Min/+ mice were treated twice with 5 mg/kg AOM, and the number of colonic tumours was increased more than 6-fold compared with controls (Table I). A total of 78 colonic tumour samples from AOM-treated mice were analysed for LOH in the *Apc* gene (Figure 1). Forty-one percent of the samples showed LOH whereas 18% were not-LOH. In control *Min* mice, 8 out of 9 (89%) colonic tumours showed LOH, whereas one sample was uncertain. A genetic LOH analysis has, to our knowledge, not previously been performed on colonic tumours from AOM-treated *Min* mice. However, immunohistochemical staining of AOM-exposed *Min* mice, by use of an antibody recognising the C-terminal of Apc, failed to show any full length Apc protein in 29 out of 30 colonic tumours (16), indicating LOH in *Apc*. Unfortunately, we were not able to classify 32 of the tumour samples from the AOM-treated mice with respect to LOH by our method of analysis. These

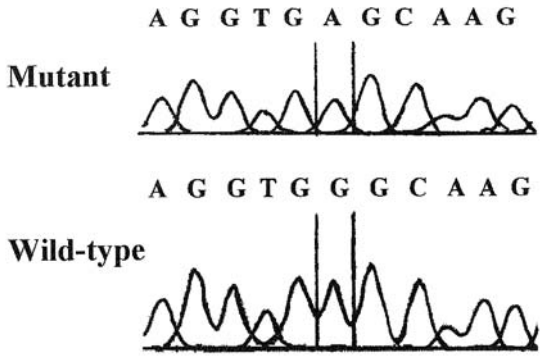

Figure 3. *The cloned mutant was sequenced according to the CEQ 8000 Genetic Analysis System. A cloned sample of the wild-type sequence is shown as a reference for comparison.*

problems with the LOH assay were unexpected, since the same method gave clear results when samples from small intestinal tumours were analysed (21, 18). One explanation could be that samples from the colonic tumours, probably to a greater extent than samples from small intestinal tumours, in addition to epithelial tissue from the tumours contained stromal tissue as well as normal crypts in the peripheral part of the tumour (25). These tissues exhibit both the *Min* and wild-type *Apc* alleles and would therefore interfere with the LOH analysis.

The tumour samples were also analyzed for nonsense mutations in the *Apc* gene by the IVSP method (Figure 1). One sample from a female AOM-treated *Min*/+ mouse showed a truncation mutation (Figure 2). Cloning and sequencing of this sample (no. 80) showed that the mutation was a G to A substitution, changing the genetic code for the amino acid tryptophan (TGG) into a stop codon (TGA) (Figure 3). The frequency of *Apc* nonsense mutations is very low, 1/78, slightly less than in tumours of *Min* mice exposed to PhIP (25/804)(6). Previously, five *Apc* truncating mutations have also been detected in colonic tumours induced by AOM in rats (26). These were predominantly C→T transitions. However, in colonic tumours induced by AOM in mice and rats, both C→T and G→A transition mutations in the β-catenin gene was found (11), most being TGGA-TGAA mutations (12, 13). The G→A mutation found in the AOM-treated Min mouse in our study was located in a similar sequence, TGGG→TGAG. The mutation identified is in concordance with mutations identified after methylation of O^6 in the DNA base guanine (9, 10). Alkylation of DNA seems to give mutations in DNA different from those of other carcinogens, such as PhIP, which makes bulky C-8-guanosine adducts in DNA (27) and primarily produced G→T mutations (6).

In this study, the mutation was located in codon 1047 (or base pair 3141) of the mouse *Apc* gene. Previously, we have found that most of the truncation mutations in *Apc* were located between codons 989 and 1156 in *Min* mice treated with the carcinogen PhIP. Therefore, even though AOM seems to give G→A transitions and PhIP G→T mutations, both carcinogens produced truncation mutations in the same area of the *Apc* gene.

Colon tumour samples not showing LOH were also analysed for β-catenin mutations. A total of 48 tumours were analysed, but no β-catenin mutations were identified. The samples were analysed by the CEQ 8000 Genetic Analysis System. Most of the samples had a heterozygote detection sensitivity of 0.15, but a few samples with more background had sensitivity up to 0.25. A possible mutation should, therefore, be identified unless the sample had a vast amount of normal tissue. Lack of β-catenin mutations in colonic tumours in our study is in agreement with another study in AOM-treated *Min* mice (8). Even though β-catenin mutations seem to be absent in AOM-treated *Min* mice, several studies have shown that AOM treatment of mice and rats produces β-catenin mutations (11-14). However, in Min mice β-catenin mutations have not been detected. The most likely explanation for this discrepancy resides in the genetic status of the *Min* mouse. *Min* mice are heterozygous for the *Min* mutation in the Apc allele and are therefore, extremely vulnerable to changes in the wild-type *Apc* allele. The dominating mechanism for spontaneous intestinal tumour formation in *Min* mice is inactivation of the wild-type *Apc* allele preferentially by LOH following somatic recombination (5, 28).

In this work, we have shown that a considerable proportion of the colonic tumours of AOM-treated *Min* mice had LOH in *Apc*. We also found this for tumours induced by PhIP in *Min* mice (6). This mechanism, therefore, seems to be the prevailing one for carcinogen-induced tumour induction in these mice. It has been reported that other DNA binding chemical carcinogens, both alkylating and compounds forming bulky adducts, may promote somatic recombination (29), hence such a mechanism is compatible with our finding of LOH in *Apc* as a major mechanism of carcinogen-induced tumourigenesis in the *Min* mouse. Further studies are needed to confirm the hypothesis of carcinogen-induced somatic recombination as a mechanism of LOH in Apc.

Another mechanism of tumour induction by AOM was nonsense mutations in the *Apc* gene. In concordance with the PhIP studies (6), these were very few. A lot of AOM-induced colonic tumours in *Min* mice did not have any clear explanation. Regarding nonsense mutations in the *Apc* gene, we only analysed codons 686-1217. Other parts of the *Apc* gene could be involved. We have previously shown that *Min* mice treated with PhIP predominantly had *Apc* truncation mutations located between codons 989 and 1156 (6), affecting the ability of the Apc protein to promote degradation of β-catenin. Five colonic tumours induced by AOM in rats showed truncation mutations between codons 1058 and 1278 (26). Nine nonsense mutations in the *Apc* gene in *N*-ethyl-*N*-nitrosurea-treated *Min* mice were located between codons 971 and 1197 (20). Mutations other than truncating mutations in *Apc* could occur and also lead to inactivation of the Apc protein. Missense mutations in the *Apc* gene may give conformation alterations in the Apc protein that renders it inactive. Another option could be mutations induced by AOM in other genes.

In conclusion, the mechanism for colonic tumour induction in AOM-treated *Min* mice is predominated by LOH of the wild-type *Apc* allele. Another mechanism is truncating mutations in the *Apc* gene also affecting β-catenin degradation, and the type of mutation observed in this study (G→A) is in concordance with other mutations observed after AOM-treatment of rodents. β-catenin mutations of intestinal tumours have not been observed in AOM-treated *Min* mice and inactivation of Apc therefore seems to be the prevailing mechanism for tumour induction in these mice.

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