

# Dominant Negative Effect of the APC<sup>1309</sup> Mutation: A Possible Explanation for Genotype-Phenotype Correlations in Familial Adenomatous Polyposis<sup>1</sup>

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## Abstract

Inactivation of the adenomatous polyposis coli (APC) gene product initiates colorectal tumorigenesis. Patients with familial APC (FAP) carry germ-line mutations in the APC gene and develop multiple colorectal adenomas and subsequent carcinomas early in life. The severity of the disease correlates with the position of the inherited APC mutation (genotype-phenotype correlation). Together with the fact that both germ-line and sporadic APC mutations cluster in the central region of the APC gene, this points to a dominant negative effect of certain APC mutants. Loss of APC function was recently shown to result in enhanced  $\beta$ -catenin-/Tcf-mediated transcription in colon epithelial cells. Here, we provide experimental evidence for a dominant negative effect of APC gene products associated with severe polyposis. Wild-type APC activity in  $\beta$ -catenin-/Tcf-mediated transcription was strongly inhibited by a mutant APC that is truncated at codon 1309. In contrast, mutant APC gene products that are associated with attenuated polyposis (codon 386 or 1465) interfered only weakly with wild-type APC activity. These results suggest a molecular explanation for the genotype-phenotype correlation in FAP patients and support the idea that colorectal tumor growth might be, in part, driven by selection for a mutation in the mutation cluster region.

## Introduction

Mutations in the APC<sup>3</sup> tumor suppressor gene are an initiating event for both familial and sporadic colorectal tumorigenesis (1, 2). The great majority of these mutations accumulate in the central region of the APC gene (MCR) and result in expression of COOH-terminally truncated proteins (3). Several authors describe evident genotype-phenotype correlations in FAP patients concerning the position of the inherited mutation and the severity of the disease. APC mutations in the first or last third of the gene are associated with an attenuated polyposis with a late onset and a small number of polyps (4–6), whereas mutations in the central region of the gene correlate with a severe phenotype of thousands of polyps at young age (7–9) and with additional extracolonic manifestations (10, 11). The molecular basis for this correlation is unclear. Nonneoplastic cells of FAP patients are expected to retain normal APC function due to the presence of one WT allele, irrespective of the mutation's position in the affected allele. According to Knudson's "two-hit" mechanism for the inactivation of tumor suppressor genes, complete loss of APC function in tumor cells should only occur if a second hit affecting the WT allele takes place. In line with this hypothesis, the WT APC allele is lost in the great majority of colorectal tumors of both sporadic and FAP

patients (2). However, clustering of APC mutations in the MCR and the correlation of the most severe FAP phenotypes with mutations in that region suggest either that this part of the gene is particularly susceptible to mutation or that tumor induction is, in part, driven by selection for a mutation in the MCR (3). The latter possibility points to dominant negative mechanisms of centrally located APC mutations that result in greater propensity for tumor growth.

Recent studies have provided some clues to understanding how mutant APC contributes to colorectal tumorigenesis. The APC protein is an integral part of a signaling pathway by complex formation with glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ),  $\beta$ -catenin (12), and the newly identified proteins axin (13) and conductin (14). WT APC mediates the degradation of  $\beta$ -catenin, thereby inhibiting CMT. Accordingly, loss of APC function results in increased levels of  $\beta$ -catenin and enhanced transcription of target genes.

Here, we sought to investigate whether certain mutant APC gene products can exert a dominant negative effect on WT APC. We expressed truncated APC proteins together with WT APC in a colorectal cancer cell line lacking endogenous WT APC and analyzed their effects on expression of a reporter gene regulated by CMT.

## Materials and Methods

**Plasmids.** The reporter plasmids pTOPFLASH, containing four copies of an optimal Tcf-LEF-1 binding element upstream of the luciferase gene, and pFOPFLASH, containing four copies of a mutant Tcf-LEF-1 binding element upstream of the luciferase gene, were kindly provided by H. Clevers (University of Utrecht, Utrecht, the Netherlands). The pRSV-lacZ vector, used as an internal control for the efficiency of the transfection, was obtained from Waltraud Ankenbauer (DKFZ, Heidelberg, Germany). The expression plasmids pCMV $\Delta$ 45Cat, containing a mutant  $\beta$ -catenin cDNA, and pCMV-APC, containing the full-length WT APC-cDNA, were kindly provided by K. W. Kinzler (Johns Hopkins Oncology Center, Baltimore, MD).

For construction of plasmids encoding truncated APC proteins, mutant cDNA fragments were generated by PCR amplification using pCMV-APC as a template. The reverse primers were designed to introduce a 2-bp deletion (at codon 386) or a 5-bp deletion (at codon 1309) and a flanking *Xho*I-restriction site for insertion into corresponding sites of pCMV-APC. For pCMV-386, the primer set MT386 (forward, 5'-CTAGGTACCTGCCAGGATATGGAA-3'; and reverse, 5'-TCCGCTCGAGTCATCAGGCTGTGAGTGAATGATGTTGTGGAGCTGC-3') was included in a 50- $\mu$ l PCR consisting of: 5 min at 93°C; 35 cycles of 30 s at 93°C, 45 s annealing at 74°C, and 30 s at 74°C; and a final extension for 10 min at 74°C. The resulting product was inserted into *Kpn*I-*Xho*I-digested pCMV-APC. To construct pCMV-1309, we included the primer set MT1309 (forward, 5'-AGTAATGCATGTGGAACCTTTGTGG-3'; and reverse, 5'-CTACTGGAGCTACTAGTTCACATCTTTTATTCTGC-3') in a 50- $\mu$ l PCR of 5 min at 93°C; 35 cycles of 30 s at 93°C, 1 min annealing at 65°C, and 3 min at 74°C; and a final extension for 10 min at 74°C. The resulting PCR fragment was inserted into pCMV-APC, which was digested with *Xho*I, blunted by Klenow and digested with *Nsi*I. pCMV-1465 was generated under the same conditions as pCMV-1309, with the reverse primer (5'-TTACTGCAGTATTAGGTCCACTCTCTCTTTTCAGC-3') introducing a 2-bp deletion at codon 1465. All constructs were confirmed by sequencing.

**Cell Lines and Transfections.** The colorectal cancer cell line SW948 was provided by the tumor collection of the German Cancer Research Center

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<sup>3</sup> The abbreviations used are: APC, adenomatous polyposis coli; MCR, mutation cluster region; FAP, familial APC; CMT,  $\beta$ -catenin-/Tcf-mediated transcription.

(Tumorbank, DKFZ). For transient transfections, SuperFect (Qiagen, Hilden, Germany) was used as a precipitation reagent according to the manufacturers instructions. For transfection of pCMVΔ45Cat,  $2 \times 10^5$  cells were cotransfected with 0.5 μg of the luciferase reporter plasmid pTOPFLASH, 0.1 μg of an internal control (pRSVlacZ), and the indicated amounts of a mutant β-catenin expression plasmid. Empty pBluescript SK+ vector (Stratagene, Heidelberg, Germany) was added to a total DNA amount of 3.0 μg. For expression of WT APC (pCMV-APC) or coexpression of mutant and WT APC,  $2 \times 10^6$  cells of SW948 were cotransfected with 1.4 μg of the reporter plasmid (pTOPFLASH or pFOPFLASH), 0.5 μg of an internal control (pRSV-lacZ), the indicated amount of WT and mutant APC expression vectors (pCMV-386, pCMV-1309, or pCMV-1465, respectively), and pBluescript-SK+ vector up to a total DNA amount of 8.6 μg. After 3 h, the supernatant was replaced by fresh medium (RPMI 1640; Life Technologies, Inc.), and the cells were incubated for 24 h.

**Reporter Gene Assay.** Cells were washed twice in PBS and lysed in 250 μl of 25 mM Tris-PO<sub>4</sub> (pH 8.0), 2 mM DTT, 2 mM *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid (CDTA), 10% glycerol, and 1% Triton X-100 by two cycles of freeze-thawing. Lysates were scraped off the culture dishes, and nonsoluble cell debris was pelleted by centrifugation. Each supernatant was used for both β-galactosidase and luciferase assays, as described (15).

**Western Blots.** Complete cell lysates were obtained by harvesting the cells in trypsin, EDTA, and PBS. Cells were pelleted, washed twice in PBS, and lysed in 2× Laemmli sample buffer at  $10^4$  cells/μl. Ten μl of the total cell lysate were separated on a vertical 3% agarose gel in 89 mM Tris base, 89 mM boric acid, 2 mM EDTA (pH 8.0), and 0.1% SDS at 90 V and transferred overnight onto a nitrocellulose membrane by capillary transfer in 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.04% SDS. The monoclonal antibody anti-APC (Ab-1; Dianova, Hamburg, Germany) was used to detect full-length and truncated APC proteins following the manufacturer's protocol.

**Results and Discussion**

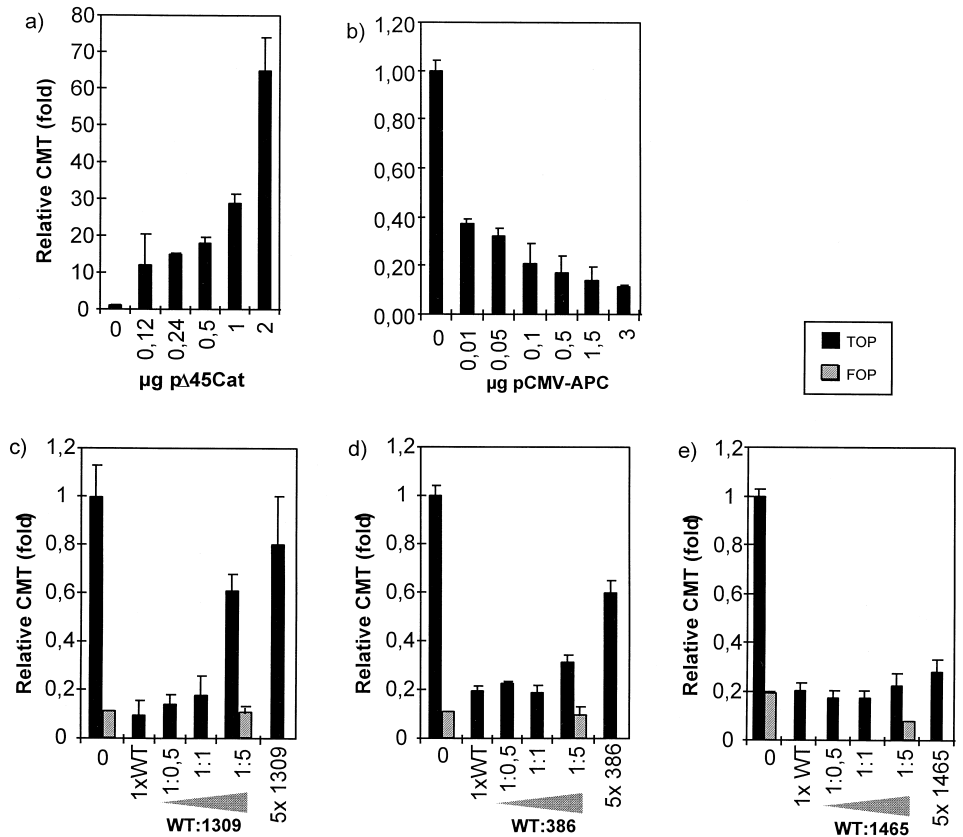
Current models explaining the mechanisms by which APC mutations predispose kindreds to FAP or initiate sporadic colorectal tumors

suggest a two-hit inactivation of both APC alleles. However, abnormal enterocyte growth in nonadenomatous intestinal tissue from *Min/+* mice and FAP patients, where WT APC is still present, has been reported (16, 17). To evaluate the hypothesis that truncated APC proteins, resulting from mutations within the MCR, might inactivate the remaining WT APC, we took advantage of a recently described CMT reporter assay. Loss of APC function results in increased amounts of free cytosolic β-catenin, which, by interaction with the T cell factor-lymphoid enhancer factor (Tcf-Lef) family, modulates expression of target genes (18, 19). Constitutive CMT has been noted in APC<sup>-/-</sup> colorectal cancer cell lines (SW480, SW620, and DLD-1) expressing only truncated APC, whereas exogenous expression of WT APC significantly reduces CMT (18, 19). Accordingly, we wished to investigate whether certain APC mutants interfere with this WT APC-dependent reduction of CMT in a dominant negative manner.

The colorectal cancer cell line SW948 was used for analysis because it contains only mutant APC truncated upstream the MCR at codon 1114. To evaluate CMT regulation in SW948 cells, we tested the ability of different expression constructs to activate luciferase expression from a Tcf-Lef-responsive reporter (pTOPFLASH). Cotransfection of SW948 cells with pTOPFLASH and increasing amounts of a mutant β-catenin construct (pΔ45Cat), which was recently reported to form a stable complex with members of the Tcf-Lef family (20), revealed a dose-dependent increase of luciferase activity (Fig. 1a). In contrast, cotransfection of a WT APC expression vector with the reporter gene reduced the basal CMT of SW948 cells to the same level as that reported previously for SW480 colorectal cancer cells (Fig. 1b; Refs. 18 and 19).

We next addressed the question of whether mutant APC alleles associated with either severe or attenuated FAP differentially affect CMT. Three APC mutants, representing naturally occurring germ-line alterations of FAP-patients, were coexpressed together with WT APC

Fig. 1. Effects of different transiently transfected constructs on CMT in SW948 cells. Twenty-four h after transfection, luciferase activity was assayed in cell lysates and normalized for β-galactosidase activity. The resulting CMT activity is expressed relative to assays containing only the reporter plasmid. a,  $2 \times 10^5$  cells were cotransfected with a Tcf-Lef responsive luciferase reporter (pTOPFLASH) and the indicated amounts of a mutant β-catenin expression plasmid (pCMVΔ45Cat). Columns, means of two independent assays; bars, SD. b-e,  $2 \times 10^6$  SW948 cells were cotransfected with pTOPFLASH and a WT APC expression plasmid (pCMV-APC; b) or the indicated molar ratios of 1.4 μg of pCMV-APC together with the indicated APC mutant (pCMV-1309, pCMV-386, or pCMV-1465, respectively). The resulting CMT was compared with that of cells transfected with only 1.4 μg of pCMV-APC (1×WT) and with that of cells containing the 5-fold molar amount of the respective mutant APC expression plasmid alone (5×1309, 5×386, and 5×1465). Columns, means of four independent assays; bars, SE. None of the APC constructs activated luciferase transcription from a modified reporter pFOPFLASH, containing mutant Tcf-Lef binding elements, which is not activated by β-catenin-Tcf-Lef complexes (■).



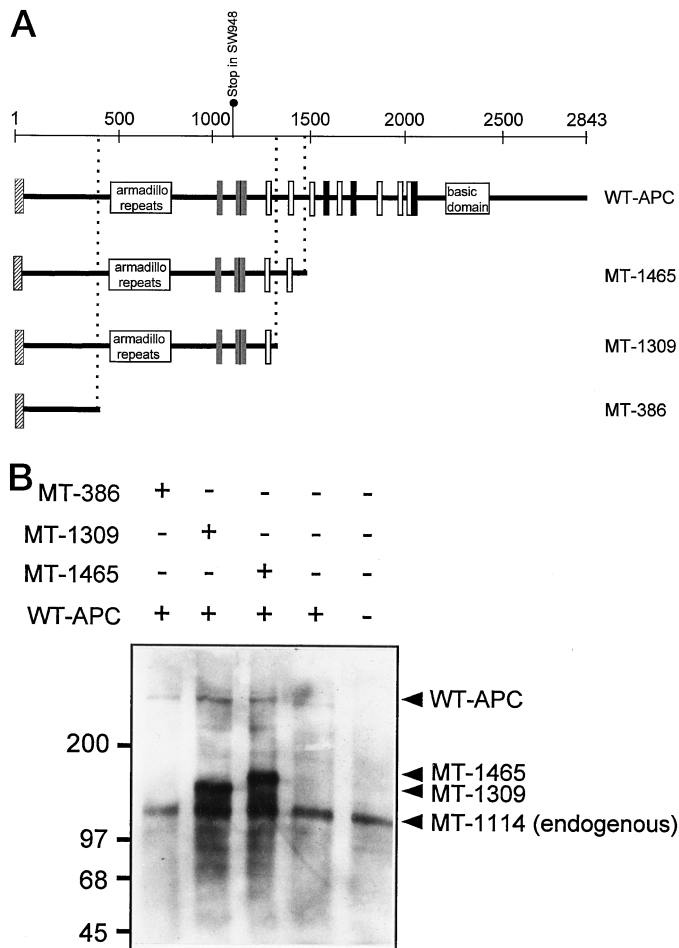


Fig. 2. *A*, scheme of the APC cDNA and the mutant APC cDNAs which were used for reporter gene assays. APC is a 2843-aa protein, containing a dimerization domain (▨), armadillo repeats, three  $\beta$ -catenin-binding regions (▣), seven 20-aa repeats (□), SAMP repeats (■), and a basic domain. *B*, expression analysis of SW948 cell lysates cotransfected with a 1:5 molar ratio (WT-APC:MT-APC). The lysates were separated on a 3% agarose gel, developed with anti-APC antibody (Ab-1), and visualized by chemoluminescence (ECL).

in SW948 cells (Fig. 2*a*). A 5-bp deletion at codon 1309 (MT-1309) leading to truncation of the protein three codons downstream is the most frequent mutation found in FAP patients with severe polyposis (21). In contrast, an attenuated FAP phenotype is associated with a 2-bp deletion in codon 386 of the APC gene (MT-386), resulting in a translation stop 12 codons downstream (Heidelberg Polyposis Registry). The third mutant, MT-1465, results from a 2-bp deletion in codon 1465 and represents a FAP phenotype characterized by a moderate number of colonic polyps accompanied by desmoid tumors. Increasing molar amounts of each mutant were cotransfected with a constant amount of WT APC and the reporter plasmid into SW948 cells. The three APC mutants clearly differed in their interference with WT APC-dependent transcription of the reporter gene. Addition of MT-1309 significantly antagonized CMT reduction, achieved by expression of exogenous WT APC in a dose-dependent manner (Fig. 1*c*). A 5-fold excess of MT-1309 over WT APC almost completely abrogated the CMT down-regulating effects of WT APC, which strongly points to a dominant negative effect of this mutant. Expression of the maximum amount of MT-1309 in the absence of any WT APC did not alter the basal constitutive luciferase activity. In contrast, MT-386 had no significant effect on WT APC-mediated reduction of CMT. Five-fold molar excess of MT-386 over WT APC resulted in a slight increase of Tcf-Lef-dependent transcription compared to that exerted

by WT APC in the absence of any exogenous mutant APC (Fig. 1*d*). However, this increase is far from the effect mediated by an equivalent molar amount of MT-1309. It is unclear why expression of the maximum amount of MT-386 without any WT APC resulted in lower CMT (~60%), compared to that in cells transfected with the reporter plasmid alone. Possibly at least part of MT-386 interferes with the endogenous mutant APC or with other components of the signaling pathway, thereby diminishing the constitutive CMT activation, mediated by the codon 1114 mutant. Likewise, analysis of MT-1465 revealed no inhibition of WT APC function (Fig. 1*e*). However, in contrast to MT-386, expression of high amounts of MT-1465 in the absence of WT APC reduced the endogenous CMT in SW948 close to that of WT APC, indicating that it can partially mediate WT APC activities.

How do different APC mutants confer differential regulation of residual WT APC activity, depending on the site of the truncating mutation? On the basis of the dimerization properties of APC, it was proposed that mutations at the extreme 5' end of the gene (*i.e.*, MT-386) presumably lead to unstable heterodimers of mutant with WT APC, thus providing sufficient levels of active WT APC dimer (6, 22). Alternatively, these small APC proteins or their corresponding transcripts might be unstable, consistent with the observation that very small APC proteins of  $M_r < 80,000$  or truncated 5' to exon 15 cannot be detected in FAP patients (23, 24). Centrally located mutations, like the one represented by MT-1309, might result in stable heterodimers that are incapable of signal transmission, or they might acquire new functions. In contrast, mutations resulting in APC proteins truncated downstream of the MCR should form stable heterodimers that are still capable of mediating partial WT APC activities.

If the APC mutants differ in transcript or protein stability, their levels in equivalently cotransfected cells should be different. We, therefore, performed Western blot analysis of SW948 cells, cotransfected with WT APC together with a 5-fold molar excess of each of the three APC mutants (Fig. 2*b*). MT-1309 and MT-1465 were present at levels that were as high as those of the endogenous truncated APC. In contrast, MT-386 was not detected, although its correct expression could be confirmed in stably transfected 293 cells (data not shown). This finding supports the idea that MT-1309 and MT-1465 both show increased stability, whereas MT-386 is unstable either on the RNA or protein level. Accordingly, MT-386 interferes only weakly with CMT suppression mediated by WT APC dimers. In concordance with our data and the model described above, MT-1309 might inactivate WT APC, thereby exerting a dominant negative effect on CMT due to its increased stability, whereas MT-1465, showing equal stability, might be capable to mediate regulation of the cytosolic  $\beta$ -catenin level at least in part. MT-1465 contains all of the 15-aa  $\beta$ -catenin-binding-sites and two of the 20-aa repeats essential for down-regulation of  $\beta$ -catenin, but it lacks the SAMP repeats, which associate with conductin to mediate  $\beta$ -catenin degradation (14). Therefore, it is conceivable that high amounts of MT-1465 titrate  $\beta$ -catenin out of the free cytosolic pool, thereby reducing CMT, although  $\beta$ -catenin degradation is diminished. In line with these data, we observed that SW948 cells stably transfected with MT-1465 grow much more slowly than cells stably expressing MT-1309 or MT-386, respectively (data not shown).

Taken together, the data presented here demonstrate, for the first time, direct evidence for a dominant negative mechanism of the 1309 mutant on a signaling pathway in human colorectal cancer cells. Contrary results were reported from transgenic mice that contained a third-copy *Apc* minigene, truncated at codon 716 in addition to two WT alleles (25). No polyp or tumor could be detected in these animals, although *Apc*<sup>716</sup> knockout mice developed numerous intestinal polyps. In light of the observation, that truncated APC, resulting

from centrally located mutations is apparently more abundant than WT APC in cell lysates of FAP patients (22), a dose dependency seems likely. The level of mutant *Apc*<sup>716</sup> expressed from the minigene might have been too low in relation to WT APC expressed from two intact alleles to exert a dominant effect. A similar dose dependency of the manifestation of a dominant negative effect was recently reported for a *hPMS2* mutation, which exerts mismatch repair deficiency only in some individuals of a family carrying the same germ-line mutation (26). Alternatively, the *Apc*<sup>716</sup> mutation may result in different signaling activity than MT-1309. In *Min* mice, another animal model for FAP with a germ-line mutation at codon 850 of the murine *Apc* homologue, a dominant negative mechanism was proposed upon intestinal cell migration. The authors described increased  $\beta$ -catenin levels and a decrease of the proliferation rate together with a decrease in apoptosis in histologically normal (*Min*/+) mucosa compared to that of +/+ animals (16). Accordingly, they hypothesized a prolonged life span for enterocytes, thereby increasing the probability of additional mutations promoting tumorigenesis. Our results link these effects to an intracellular signaling mechanism. Although, the overall mechanisms predisposing to multiple tumor formation might be different in mice and humans, alterations in the proliferation rate and/or apoptosis of the colorectal epithelium due to dysregulation of CMT of target genes, might be common to both species. Enhanced proliferation of normal intestinal mucosa was also described in patients with severe polyposis (17).

In summary, our study should greatly help in understanding the genotype-phenotype relationship observed in FAP patients. In addition, the clustering of APC mutations found in sporadic colorectal lesions could be ascribed to dominant negative mechanisms preceding further steps of colorectal tumorigenesis. It remains to be investigated whether the observed mechanisms affecting CMT are restricted to the APC-1309 mutation. We are currently analyzing a panel of further chain-terminating APC mutations within the MCR for their interference with WT APC and their effects on CMT to evaluate their tumorigenic potential.

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