Transactivation of the Multidrug Resistance 1 Gene by T-Cell Factor 4/β-Catenin Complex in Early Colorectal Carcinogenesis^1

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Abstract

The mutational inactivation of a tumor suppressor gene, adenomatous polyposis coli (APC), results in the accumulation of cytoplasmic β-catenin protein and the activation of T-cell factor (TCF)/lymphoid enhancer factor transcriptional factors. A colorectal carcinoma cell line, DLD-1, was engineered to suppress transactivation by the TCF4/β-catenin complex in a dominant-negative manner under the strict control of the tetra-cycline regulatory system. A large-scale comparison of the expression profiles, using two-color fluorescence hybridization of cDNA microarray, led to the identification of MDR1 as a target gene of the TCF4/β-catenin complex. Luciferase reporter and gel retardation assays revealed the TCF4/β-catenin responsive elements in the promoter of the human MDR1 gene. Corresponding to the accumulation of β-catenin, expression of the MDR1 gene product was steadily up-regulated in adenomas and adenocarcinomas of 10 patients with familial adenomatous polyposis. In combination with cell proliferative activities of c-myc and cyclin D1, MDR1 may initiate colorectal tumorigenesis by suppressing cell death pathways programmed in intestinal epithelial cells.

Introduction

The germ-line mutation of a tumor suppressor gene, APC,^3 causes FAP syndrome (1, 2). Nearly 100% of FAP patients suffer from numerous adenomatous polyps of the colon and rectum during their second or third decades of life and, if not treated adequately, progression of adenoma into invasive adenocarcinoma is virtually inescapable. In addition to this high degree of predisposition in FAP patients, 80–90% of patients with sporadic colorectal cancer harbor APC mutations (3). The mutation and loss of heterozygosity of APC occurs as the earliest event in hereditary and sporadic adenoma-carcinoma transition has still not been established. β-catenin is a mediator of the Wingless and Wnt signaling pathways (8). By forming a complex with TCF/LEF DNA-binding proteins, β-catenin acts as a transcriptional coactivator (9). In fact, colorectal cancer cell lines with mutated β-catenin or APC show constitutively active TCF/LEF transcription (10). Thus far, genes including c-myc, cyclin-D1, matrilysin, TCF1, and peroxisome proliferator-activated receptor δ have been reported to be the target genes of the TCF/LEF and β-catenin transcriptional complex (11–14). The whole picture of gene expression profiles initiating early colorectal carcinogenesis is, however, still obscure. TCF4 is a TCF/LEF family member expressed in intestinal epithelia (10). TCF4 lacking an NH2-terminal β-catenin binding site was reported to suppress specifically transactivation by the TCF4/β-catenin complex in a dominant-negative manner (10). In this study, a colorectal carcinoma cell line was engineered to induce the truncated dominant-negative TCF4 protein under the strict control of the tetra-cycline-regulatory system (15). This cell line enabled us to pinpoint precisely the cellular and molecular changes after the inactivation of TCF4. We report here that the human MDR1 gene is transcriptionally regulated by the TCF4/β-catenin complex and that aberrant expression of MDR1 occurs as an early event in colorectal carcinogenesis.

Materials and Methods

Expression Constructs and Cell Lines. AU1-tagged TCF4β cDNA, lacking a 30-amino acid β-catenin binding site in its NH2 terminus (nucleotides 398-2138 in accession number Y11306), was amplified by PCR and subcloned into pTRE (Clontech), pEGFP-C1 (Clontech), or pCR3.1 (Invitrogen) to make pTRE-TCF4βN30, pEGFP-TCF4βN30, or pCR3.1-TCF4βN30, respectively. The compositions of all of the constructs in this study were confirmed by restriction endonuclease digestion and by sequencing. Details of the procedures used for plasmid construction are available on request.

A colon cancer cell line, DLD-1, was obtained from the Riken Cell Bank. DLD-1 was double-transfected sequentially with regulatory pTet-ON (Clontech) and responsive pTRE-TCF4βN30 plasmids using LipofectAMINE PLUS (Life Technologies, Inc.). Three stable clones, DLD-1 Tet-ON TCF4βN30-1, -5, and -7, all of which were capable of inducing truncated TCF4βN30 in the presence of 2 μg/ml doxycycline (Sigma), were isolated. The integration of empty pTRE plasmid DNA was confirmed by PCR in four mock clones, DLD-1 Tet-ON control-A, -B, -C, and -D.

Two other colorectal cancer cell lines, SW48 and SW480, were purchased from the American Type Culture Collection. SW48 has a missense mutation at codon 1338 and the loss of the other allele (7).

Antibodies. Anti-TCF4β rabbit polyclonal antibody was raised against a keyhole limpet hemocyanin-conjugated synthetic peptide, CYKVKAASAH-PLQMEAY. Monoclonal murine antibody against β-catenin and an irrelevant control monoclonal antibody of the same isotype (IgG1) were purchased from Transduction Laboratories. Monoclonal antibodies against P-glycoprotein,
C494 and C219, were purchased from Dako Corporation. C219 is known to react with both MDR1 and MDR3 gene products (16).

**Northern Blot and Western Blot Analyses.** Total RNA (15 μg/lane) was fractionated by electrophoresis and transferred to Hybond N (Amersham). Hybridization was performed by using 32P-radiolabeled cloned cDNA fragments of MDR1 (nucleotides 382–912, accession number M14758) and TCF4B (nucleotides 546-2015), as described previously (17). The quality and quantity of electrophoresed RNA was determined by hybridization with glyceraldehyde-3-phosphate dehydrogenase cDNA (Clontech).

Western blot analyses were performed essentially as described previously (17). The quality and quantity of electrophoresed protein was determined by reblotting with anti-Na/K-ATPase (a3 subunit) monoclonal antibody (Affinity Bioresearch; Ref. 5).

**cDNA Microarray Screening.** mRNA was prepared from DLD-1 Tet-ON TCF4BΔN30–7 cells treated with 2 μg/ml doxycycline for 48 h or from untreated cells. Two-color fluorescence hybridization of cDNA microarray was performed by Genome Systems, Inc. Two cDNA samples, labeled either with Cy3 or Cy5 fluorescent dyes, were applied competitively to a single UniGene V microarray, containing over 7000 human cDNA mapped in the UniGene database.

**Immunohistochemistry.** Ten FAP patients were selected from the surgical pathology panel of National Cancer Center Central Hospital, 8 of whom have been described previously (5). Formalin-fixed and paraffin-embedded colorectal tissues containing adenoma (all 10 cases) and carcinoma (5 of 10 cases) were stained using the avidin-biotin complex method as described previously (5, 16).

**Reporter Constructs and Luciferase Assay.** A fragment of the MDR1 gene promoter (~220/-31; Ref. 18) was fused upstream of the firefly luciferase gene in pGL3-basic (Promega). The nested deletion mutants, listed in Fig. 4D, were generated from this construct by digestion with restriction endonucleases, blunting with T4 DNA polymerase and recircularizing with T4 DNA ligase.

Three copies of the 15-bp optimal TCF/LEF motif, CCCCTTGATCT-TACC, or the mutant motif, CCCCTTGCTCT-TACC, (10) were cloned into the MluI/BglII site of pGL3-promoter (Promega; pGL3-TOP and pGL3-FOP, respectively).

Cells were transiently transfected with reporters using LipofectAMINE PLUS in triplicate, and 48 h later luciferase activity was measured using a Luciferase assay system (Promega).

**Gel Retardation Assay.** Nuclear extracts were prepared as described previously (10). pCR3.1-TCF4BΔN30 or control pCR3.1 was transcribed and translated in *vitro* using the TNT reticulocyte lysate system (Promega). Double-stranded oligonucleotide probes were 32P-labeled using T4 polynucleotide kinase (Takara Shuzo). The sequences of probes used for gel retardation assays in this study are listed in Fig. 4A. After incubating radiolabeled probes with nuclear extracts or *in vitro* translation products, in the presence or absence of excess cold competitors, for 20 min under the conditions described previously (10), the samples were incubated for an additional 20 min with antibodies and then electrophoresed through nondenaturing 4% polyacrylamide gel run in 0.25× TBE at room temperature.

**Results**

**Establishment of DLD-1 Tet-ON TCF4BΔN30.** A colon cancer cell line, DLD-1, has a mutation at codon 1416 of the APC gene and loss of the other allele, resulting in constitutively active TCF/LEF (7). Using a tetracycline regulation system, we established three clones that were capable of inducing dominant-negative TCF4B (Fig. 1A) and four mock clones from DLD-1. In the presence of doxycycline, DLD-1 Tet-ON TCF4BΔN30 clones induced truncated TCF4B protein (Fig. 1B) and mRNA (Fig. 2A) in a time-dependent manner, but DLD-1 Tet-ON control cells did not.

Transient transfection and luciferase assays (Fig. 1C) revealed that DLD-1 Tet-ON TCF4BΔN30 clones maintained constitutive TCF/LEF activity, as shown by the high luciferase activity driven by the pGL3-TOP reporter carrying trimerized optimal sequences for TCF/LEF and the low activity driven by the mutant reporter, pGL3-FOP (uninduced, Fig. 1C). In parallel with the induction of dominant-negative TCF4B, the activity driven by pGL3-TOP was diminished to the level of pGL3-FOP within 36 h (Fig. 1C). The luciferase activity driven by pGL3-FOP was unaffected by the induction of dominant-negative TCF4B. The same doxycycline treatment did not affect the luciferase activity driven by pGL3-TOP and pGL3-FOP in mock clones (data not shown).

**Identification of MDR1 as a Target Gene of the TCF4/β-Catenin Complex.** We compared the gene expression profiles of DLD-1 Tet-ON TCF4BΔN30–7 cells treated with 2 μg/ml doxycycline for 48 h and those of untreated cells using two-color fluorescence hybridization to cDNA microarray. Among over 7000 genes examined, the expression of the MDR1 gene was found to be significantly down-regulated upon induction of the dominant-negative TCF4B. Northern blot (Fig. 2A) and Western blot (Fig. 2B) analyses confirmed that the induction of dominant-negative TCF4B suppressed the expression of the MDR1 gene. The MDR1 cDNA probe used in this study was selected from an unconserved region between MDR1 and MDR3 to avoid cross-hybridization to cDNA microarray. Among over 7000 genes examined, the expression of the MDR1 gene was found to be significantly down-regulated upon induction of the dominant-negative TCF4B.

**Ablerrant Expression of MDR1 in Early Colorectal Carcinogenesis of FAP Patients.** We examined colorectal tissues from 10 FAP patients immunohistochemically. In all 10 cases, numerous adenomatous polyps were observed throughout the resected colorectal specimens. Invasive adenocarcinoma was observed in five cases. Serial sections were stained with anti-β-catenin and anti-P-glycoprotein (C494; Ref. 16) monoclonal antibodies to explore their correlation.
β-Catenin was stained intensely in differentiated epithelial cells lining the luminal surface of nonneoplastic mucosa (Fig. 3C). Adenoma and adenocarcinoma cells showed intense immunohistochemical reactivity with anti-β-catenin antibody compared with adjacent nonneoplastic epithelial cells (Fig. 3, A and C), as described previously (5). The cells that stained intensely with anti-β-catenin antibody also overexpressed the MDR1 gene product in neighboring serial sections (Fig. 3, B and D). The glands that stained intensely with the two antibodies were almost completely matched to each other. The MDR1 gene product was localized along the entire cell membrane in adenoma cells (Fig. 3, B, D, and F), as described previously (5). The addition of excess TOP (Lane 5) or canonical (Lane 7) competitors abolished the binding, but the addition of FOP (Lane 4) or mutant (Lane 6) did not. The addition of anti-β-catenin antibody resulted in specific retardation of the latter band (arrowhead, Lane 7) confirming the presence of β-catenin in the complex.

Using products translated in vitro from the T7 promoter in pCR3.1-TCF4BΔN30, we readily detected single retarded bands in the canonical probes 1, 2, 3, and 5 (Fig. 4B, Lane 1) but not in 4, 6, and 7. The faster bands indicated the binding of TCF/LEF protein to the radiolabeled probe, and the slower faint bands indicated the binding of TCF/LEF and β-catenin complex, as previously described (7, 10). The addition of excess TOP (Lane 3) or canonical (Lane 5) cold competitors abolished the binding, but the addition of FOP (Lane 4) or mutant (Lane 6) did not. The addition of anti-β-catenin antibody resulted in specific retardation of the latter band (arrowhead, Lane 7) confirming the presence of β-catenin in the complex.

Using products translated in vitro from the T7 promoter in pCR3.1-TCF4BΔN30, we readily detected single retarded bands in the canonical probes 1, 2, 3, and 5 (Fig. 4C, Lane 1) but not in 4, 6, and 7. TCF4BΔN30 contains an intact high mobility group-box DNA-binding domain (Fig. 1A). The addition of excess TOP (Lane 5) or canonical (Lane 7) competitors abolished the binding, but the addition of FOP (Lane 6) or mutant (Lane 8) did not. The MDR1 promoter luciferase construct containing −2028/+31 responded to cotransfection with dominant-negative TCF4B (Fig. 4D). The serial deletion of reporter constructs (Fig. 4D, left) reduced the responsiveness and the removal of −2028/−229 (which excludes all possible TCF/LEF binding sites but still contains a basic promoter) abolished the responsiveness (Fig. 4D, right).

**Discussion**

The MDR1 gene was originally identified as an overexpressed and amplified gene in MDR cells (20). The MDR1 gene encodes a transmembrane protein that transports structurally different hydrophobic chemotherapeutic agents outward in an energy-dependent manner. The overexpression of the MDR1 gene after chemotherapy is one of the major causes of drug resistance. The product of the MDR1 gene, P-glycoprotein, is expressed physiologically in various tissues including the blood-brain barrier, the adrenal gland, proximal renal tubules, hepatocytes, and intestinal epithelia (16, 21). The murine knockout of the MDR1 homologue, mdr1a, resulted in disturbed secretory functioning of the blood-brain barrier and increased sensitivity to drugs (21), but the physiological functions and natural substrates in intestinal epithelia remain unknown.

In this study, we established a colorectal carcinoma cell line capable of inducing the truncated dominant-negative TCF4 protein under the strict control of the tetracycline regulatory system. Through a large-scale comparison of >7000 genes by two-color fluorescence hybridization of cDNA microarray, MDR1 was found to be transcriptionally down-regulated after the inactivation of TCF4. Aberrant expression of MDR1, concomitant with the accumulation of β-catenin, occurs even in small precancerous lesions of FAP patients. The promoter of the MDR1 gene contains multiple TCF4-binding sequences, concluding that MDR1 is a direct target gene of the TCF4/β-catenin transcriptional complex.

Besides adenoma and carcinoma, β-catenin accumulates in terminally differentiated cells lining the luminal surface of nonneoplastic intestinal mucosa (Fig. 3C, left), implying a physiological role of the TCF4/β-catenin complex in intestinal cell differentiation. Treatment with a differentiating agent, sodium butyrate, up-regulates the TCF/LEF activity of colorectal cancer cell lines (22), and consistently

**Fig. 2. Regulation of MDR1 by the TCF4/β-catenin complex.** A, Northern blot analysis. Total RNA (15 μg) extracted from DLD-1 Tet-ON TCF4BΔN30−1 cells untreated (U) or treated with 2 μg/ml doxycycline for 12, 24, or 48 h, DLD-1 Tet-ON TCF4BΔN30−5 cells untreated (U) or treated with 2 μg/ml doxycycline for 48 h, DLD-1 Tet-ON TCF4BΔN30−7 cells untreated (U) or treated with 2 μg/ml doxycycline for 48 h, and DLD-1 Tet-ON control-A cells untreated (U) or treated with 2 μg/ml doxycycline for 48 h were fractionated and blotted with TCF4B (top), MDR1 (middle), or glyceraldehyde-3-phosphate dehydrogenase (G3PDH; bottom) cDNA. B, Western blot analysis. Protein (60 μg) extracted from DLD-1 Tet-ON TCF4BΔN30−7 cells untreated (U) or treated with 2 μg/ml doxycycline for 12, 24, or 48 h and DLD-1 Tet-ON control-A cells, untreated (U) or treated with 2 μg/ml doxycycline for 48 h, were fractionated and blotted with anti-P-glycoprotein monoclonal antibody, C219 (top), or anti-Na+/K+-ATPase (α3 subunit) monoclonal antibody (bottom; Ref. 5).

TCF4/β-Catenin-responsive Elements in the MDR1 Promoter.

The registered sequence of the human MDR1 gene promoter (accession number L07624; Ref. 18) contained a cluster of seven consensus TCF/LEF-binding motifs, CTTTGCA/TAT (11), within 2.0 kb precedent the transcription initiation site (Fig. 4A). Double-stranded 15-bp oligomer probes containing these core sequences (underlined) and their flanking sequences were examined for binding to the TCF4/β-catenin complex in gel retardation assays. These canonical oligomers (Fig. 4A, left) and corresponding mutant oligomers (Fig. 4A, right) were designated 1–7 and 1mt–7mt, respectively. An optimal 15-bp TCF/LEF-binding sequence (TOP) and its mutant (FOP), described by Korinek et al. (10), were used as experimental controls (data not shown) and cold competitors. The representative autoradiograms are shown in Fig. 4, B and C.

Using nuclear extracts from SW480 cells, we readily detected two retarded bands in probes 1, 2, 3, and 5 (Fig. 4B, Lane 1) but not in 4, 6, and 7. The faster bands indicated the binding of TCF/LEF protein to the radiolabeled probe, and the slower faint bands indicated the binding of TCF/LEF and β-catenin complex, as previously described (7, 10). The addition of excess TOP (Lane 3) or canonical (Lane 5) cold competitors abolished the binding, but the addition of FOP (Lane 4) or mutant (Lane 6) did not. The addition of anti-β-catenin antibody resulted in specific retardation of the latter band (arrowhead, Lane 7) confirming the presence of β-catenin in the complex.

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sodium butyrate has been shown to enhance the expression of MDR1 (23). The activation of the MDR1 promoter by sodium butyrate has been shown to be mediated by the inhibition of histone deacetylases (24). The p300/CBP acetyltransferases function as transcriptional coactivators of β-catenin (25).

Transactivation of MDR1 by the accumulated β-catenin seems not to be limited to colorectal carcinogenesis. Mutational activation of β-catenin is reported to be frequent in chemically induced adenomatous and carcinomatous liver nodules (26). Increased expression of the mdr1 gene has been shown in similar experimental preneoplastic and neoplastic conditions (27). Overexpression of P-glycoprotein has also been reported in adenomatous hyperplasia of human liver (28).

The way in which overexpression of MDR1 is involved in colorectal carcinogenesis was not determined in the current study. The MDR1 gene product itself seems not to have transforming activity. Rapid turnover is a characteristic of intestinal epithelia. The intestinal stem cells continuously proliferate and give rise to a variety of committed cells. The integrity of the intestinal epithelium is maintained by proportional cell death of terminally differentiated cells. The failure of proper cell death disturbs the homeostatic turnover and may allow secondary genetic changes such as oncogenic activation of K-ras (3). A colorectal cancer cell line HT29 has been shown to induce apoptosis by restoring wild-type APC (29). MDR1, a downstream target of the APC pathway, may suppress cell death pathways programmed in intestinal cells by secreting endogenous or xenobiotic toxic substances into the intestinal lumen or by mediating a more

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Fig. 3. Immunohistochemical analyses of colorectal neoplasms of FAP patients. Immunoperoxidase staining with anti-β-catenin monoclonal antibody (A and C) and with anti-MDR1 gene product monoclonal antibody, C494 (B, D, E, and F). A and B, a small adenomatous polyp, 3 mm in size, with mild to moderate atypia, in serial sections (×40). Arrowheads, nonneoplastic glands. C and D, adenoma with moderate atypia (right) and adjacent nonneoplastic mucosa (left) of the colon in serial sections (×200). E, a high-power view of adenoma with moderate atypia (×400). F, invasive adenocarcinoma (right) and adjacent nonneoplastic glands (arrowheads, left) of the colon (×100).
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generic antiapoptotic function (30). The MDR1 gene product, P-glycoprotein, protects cells against a wide variety of caspase-dependent death stimuli, including FasL, tumor necrosis factor, and UV irradiation (30).

Identification of MDR1 as a target of the TCF4/-catenin complex is of clinical importance, because several compounds that antagonize MDR1, such as verapamil and cyclosporin A, have been developed to treat MDR cancers (31). The preventive efficacy of these compounds may be worth examining in multiple intestinal neoplasia (Min) mice (32) and subsequently in FAP patients.

Acknowledgments

We thank Dr. H. Clevers and Dr. M. van de Watering for providing sequences of pTOP-FLASH and pFOP-FLASH and Dr. J. Yokota for useful suggestions about a tetracycline regulatory system.

References


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*Cancer Res* 2000;60:4761-4766.

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