Mitomycin C Resistance Induced by TCF-3 Overexpression in Gastric Cancer Cell Line MKN28 Is Associated with DT-diaphorase Down-Regulation

Norihiko Sagara and Masaru Katoh

Abstract

TCF transcription factors are mediators of the WNT signaling pathway and are antagonized by the transforming growth factor β signaling pathway. Here human TCF-3 has been cloned and characterized. Differential expression analyses of TCF genes in gastric cancer revealed that TCF-1 was expressed in most cases of primary gastric cancer at almost the same level as in normal gastric mucosa and that TCF-3 was occasionally up-regulated in primary gastric cancer. The TCF-3 expression vector was transfected to gastric cancer cell line MKN28 to establish stable transformants. Three independent MKN28 transformants overexpressing TCF-3 showed about 8-fold resistance to mitomycin C (MMC; IC₅₀ 2.4 μg/ml) compared with MKN28 vector transfectants (IC₅₀ = 0.3 μg/ml). Among the 10 drug resistance-associated genes examined in this study, the DT-diaphorase (DTD) gene was down-regulated in three MKN28 transformants overexpressing TCF-3. DTD mRNA was also down-regulated in primary gastric cancer with TCF-3 up-regulation. In addition, DTD protein was down-regulated in three MKN28 transformants overexpressing TCF-3 compared with MKN28 vector transfectants. DTD is implicated in the activation of MMC in target cells, and DTD down-regulation explains MMC resistance. MMC resistance induced by TCF-3 overexpression is probably due to DTD down-regulation, which might provide a possible target for new therapy of drug-resistant gastric cancer.

Introduction

TCF/LEF transcription factors with the HMG box are implicated in the WNT signaling pathway and are antagonized by the transforming growth factor β signaling pathway (1, 2). With WNT signaling activation, β-catenin is stabilized and translocated to the nucleus to associate with TCFs. The TCF-β-catenin complex regulates the transcription of target genes such as c-myc and cyclin D1 (3, 4). TCF-1 and LEF-1 are up-regulated in hematological malignancies, whereas TCF-4 is up-regulated in colorectal cancer (3, 4). Here we have cloned and characterized human TCF-3 (5). Differential expression analyses on all members of the human TCF gene family in gastric cancer indicated that TCF-3 was occasionally up-regulated in primary gastric cancer and also indicated that TCF-1 was expressed in most cases of primary gastric cancer at almost the same level as in normal gastric mucosa. To further investigate the biological role of TCF-3 up-regulation in gastric cancer, the TCF-3 expression vector was transfected to MKN28 cells with cytosolic β-catenin stabilization and without detectable endogenous TCF-3 mRNA. In three independent MKN28 transformants overexpressing TCF-3, resistance to MMC was increased 8-fold compared with MKN28 vector transfectants. DTD mRNA was down-regulated in MKN28 transformants overexpressing TCF-3 and in primary gastric cancer with TCF-3 up-regulation. The DTD protein, which is implicated in MMC activation (5, 6), was also down-regulated in three independent MKN28 transformants overexpressing TCF-3. This is the first report demonstrating the association between TCF-3 overexpression and MMC resistance.

Materials and Methods

RNA Extraction. Poly(A)⁺ RNAs were extracted from the gastric cancer cell lines OKAJIMA, TMK1, MKN7, MKN28, MKN45, MKN74, and KATO-III with the FastTrack 2.0 Kit (Invitrogen). Total RNAs were extracted with Isogen (Nippon Gene) from a primary gastric cancer obtained during surgery at National Cancer Center Hospital.

Northern Blot Analyses. Northern blot filters containing 2 μg of poly(A)⁺ RNA or 20 μg of total RNA for each lane were prepared and hybridized with [α-³²P]dCTP-labeled probes as described previously (7).

cDNA Library Screening. The human fetal lung cDNA library in Agt10 (Clontech Laboratories) was screened with the TCF-3 probe as described previously (7).

Construction of the TCF-3 Expression Vector. CF310 and CF307 plasmids containing overlapping TCF-3 cDNAs were digested with BamHI, and a 0.9-kb BamHI fragment of CF307 was ligated to a 4.0-kb BamHI fragment of CF310, and an 1860-bp EcoRI fragment containing the total ORF of TCF-3 (TCF-3-ORF) was ligated to pcDNA3.1(+) expression vector (Invitrogen).

Establishment of Stable Transformsants Overexpressing TCF-3. The TCF-3 expression vector (TCF-3-ORF/pcDNA3.1) or the empty pcDNA3.1 vector was transfected to gastric cancer MKN28 cells using Superfect Transfection Reagent (Qiagen). After G418 selection (600 μg/ml), several stable transformants were established by the cylinder technique. Five transformants established with the empty pcDNA3.1 vector were mixed together and are described as the MKN28 vector transfectants. Poly(A)⁺ RNAs were extracted from 16 transformants established with the TCF-3 expression vector, and transformants overexpressing TCF-3 were further selected by Northern blot analysis using the TCF-3-ORF cDNA fragment as a probe.

Cell Proliferation Assay. Cells were plated on a 96-well plate (3 × 10⁴ cells/well) in triplicate and cultured in a CO₂ incubator at 37°C for 24 h. Anticancer drugs such as MMC were added to each well, and cells were cultured for 48 h. Ten μM of TetraColor One reagent containing tetratizolium monosodium salt (Seikagaku Corp.) were added to each well, and cells were incubated for 2 h. Absorbance at 490 nm was measured with a reference wavelength at 595 nm in the microplate reader.

Multiplex cDNA-PCR. Poly(A)⁺ RNAs were reverse-transcribed with the pd(N)₅ random hexamer primer by using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech). Aliquots of the cDNAs corresponding to 40 ng of poly(A)⁺ RNAs were used for the subsequent multiplex PCR with KOD...
domain, the HMG box DNA-binding domain, the nuclear translocation signal, and two putative CtBP binding sites (Fig. 1B).

Among the human TCF family, TCF-3 is most homologous to TCF-4 (58%). TCFs are homologous in the HMG box and in the β-catenin binding domain. Amino acid identity in the HMG box is as follows: (a) TCF-3 versus TCF-4, 93%; (b) TCF-3 versus TCF-1, 92%; and (c) TCF-3 versus LEF-2, 93%. TCFs are divergent in the COOH-terminal region. TCF-3 and TCF-4 contain two CtBP binding sites, but TCF-1 and LEF-1 lack CtBP binding sites.

**Expression of TCFs in Gastric Cancer.** To investigate the differential expression pattern of members of the human TCF family, each TCF-specific probe was synthesized. The CF3S probe (nucleotides 2237–2721 of human TCF-3 cDNA) detected the 3.0-kb TCF-3 mRNA. The CF1S probe (nucleotides 110–549 of human TCF-1 cDNA) detected the 4.0- and 2.0-kb TCF-1 mRNAs. The CF2S probe (nucleotides 8–448 of human LEF-1 EST AA306770) detected the 4.2- and 2.8-kb LEF-1 mRNAs. The CF4S probe (nucleotides 2093–2362 of human TCF-4 cDNA) detected the 3.0-kb TCF-4 mRNA (Fig. 2). Among human gastric cancer cell lines, TCF-3 was expressed in TMK1 and MKN45. TCF-1 was expressed in all gastric cancer cell lines examined. LEF-1 was expressed in OKA JM1 and MKN74.

**Table 1 List of PCR primers**

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<th>Primer</th>
<th>Nucleotide sequence</th>
<th>Nucleotide position</th>
<th>Accession no.</th>
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<td>PD0U</td>
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<td>J03934</td>
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<tr>
<td>PD10D</td>
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<td>1420–1399 of DTD</td>
<td>S09649</td>
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<tr>
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<td>488–509 of GST</td>
<td>X06547</td>
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<tr>
<td>PD2D</td>
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<td>710–687 of GST</td>
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<td>2067–2087 of P450R</td>
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<tr>
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<tr>
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<td>4743–4762 of MRPI</td>
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<td>5154–5135 of MRPI</td>
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<td>PD9U</td>
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<td>4852–4872 of MRPI</td>
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<td>BACTD</td>
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<td>943–924 of β-actin</td>
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**Plus DNA polymerase (Toyobo), a pair of the target gene primers (0.3 μM each), and a pair of the β-actin primers (0.03 μM each; Table 1).**

**Western Blot Analysis.** Twenty μg of total cell lysates were electrophoresed on the SDS-PAGE gel and blotted to the polyvinylidene difluoride membrane (Millipore). The filter was incubated with anti-β-actin antibody. After further washing, the filter was incubated with horseradish peroxidase-labeled secondary antibody. After further washing, immunoreactivity was detected by using the enhanced chemiluminescence Western blotting detection system (Amer sham Pharmacia Biotech).

**Results**

**Isolation of Human TCF-3 cDNAs.** Two human ESTs homologous to the mouse Tcf-3 (8) were identified by the BLAST program. Sense primer PTCF3U (5′-CAAGGGCAGGCTCAGTACTGC-3′) and antisense primer PTCF3D (5′-CCAGAGTGGCTCAATATTGACC-3′) were designed on EST R54923 and EST H43023, respectively. CF3M cDNA was isolated by cDNA-PCR with PT CF3U and PT CF3D primers from human small intestine poly(A)⁺ RNA. Because the amount of mRNA hybridized to the CF3M probe was relatively large in human fetal lung (data not shown), the human fetal lung cDNA library in Agt10 (Clontech Laboratories) was screened with CF3M. Sixteen of 7.5 × 10⁵ clones were isolated. Overlapping TCF-3 cDNAs spanning a total of 2778 nucleotides contain a 74-bp 5′ noncoding region, a 1767-bp ORF, and a 938-bp 3′ noncoding region (Fig. 1A).
TCF-4 was expressed in TMK1 (Fig. 2A). Among human primary gastric cancer, TCF-1 was expressed in most cases of primary gastric cancer at almost the same level as in normal gastric mucosa, whereas TCF-3 was occasionally up-regulated in primary gastric cancer (Fig. 2B).

Overexpression of TCF-3 in MKN28 Cells Results in MMC Resistance. TCF-3 mRNA was not detected in MKN28 cells (Fig. 1B), and TCF-3 expression vector was transfected to MKN28 cells. TCF-3 was overexpressed in three independent MKN28 transformants, T5, T6, and T10 (Fig. 3A).

Resistance of MKN28 transformants to several anticancer drugs used for clinical treatment of gastric cancer patients was analyzed by the cell proliferation assay using TetraColor One. Although MKN28 transformants did not show any change in sensitivity to Adriamycin, cisplatinum, and 5-fluorouracil (data not shown), three independent MKN28 transformants overexpressing TCF-3 showed an 8-fold resistance to MMC (IC$_{50}$, 2.4 µg/ml) compared with MKN28 vector transfectants (IC$_{50}$, 0.3 µg/ml; Fig. 3C). These results were confirmed twice by the same experiments.

Down-Regulation of DTD in MKN28 Transformants Overexpressing TCF-3. To elucidate the mechanism of increased MMC resistance in MKN28 transformants overexpressing TCF-3, the mRNA level of the genes potentially involved in MMC resistance was investigated by multiplex cDNA-PCR. Among drug resistance-associated genes including DTD, P-glycoprotein (P-gp50R) (NADPH:cytochrome P450 reductase), GST-π (glutathione S-transferase), MRPl, MRP2, MRPl, MRP3, MRP4, MRP5, PRL6, and MDR1 (5, 9–17), the expression level of DTD mRNA was decreased in MKN28 transformants overexpressing TCF-3 (Fig. 3D). Northern blot analysis demonstrated that the DTD mRNA was down-regulated in three independent MKN28 transformants overexpressing TCF-3 as compared with MKN28 vector transfectants (Fig. 3A). DTD mRNA was also down-regulated in primary gastric cancer with TCF-3 up-regulation (data not shown). Finally, down-regulation of the DTD protein in three independent MKN28 transformants overexpressing TCF-3 was confirmed by Western blot analysis (Fig. 3B).

Discussion

Human TCF-3 encoding the HMG box transcription factor with the β-catenin binding domain, the nuclear translocation signal, and two CtBP binding sites (Fig. 1) was cloned and characterized in this study. TCF-3 is most homologous to TCF-4. TCF-3 and TCF-4 contain two CtBP binding sites in the COOH-terminal region, but TCF-1 and LEF-1 lack CtBP binding sites (Fig. 1B).

![Fig. 2. Differential expression of TCF genes in human gastric cancer. A. gastric cancer cell lines. B. primary gastric cancer. Northern blot filters containing 2 µg of poly(A)$^+$ RNA (cell lines) or 20 µg of total RNA (surgical specimen) were hybridized with CF3S (nucleotides 2237–2721 of human TCF-3 cDNA), CF1S (nucleotides 110–549 of human TCF-1A cDNA), CF2S (nucleotides 8–448 of human LEF-1 EST AA306770), or CF4S (nucleotides 2093–2362 of human TCF-4 cDNA).](image)

![Fig. 3. MMC resistance and DTD down-regulation in MKN28 cells overexpressing TCF-3. A. V, a mixture of five MKN28 vector transfectants. T5, T6, and T10, three independent MKN28 transfectants overexpressing TCF-3. A. Northern blot analyses on TCF-3 and DTD. B. The TCF-3-ORF probe (nucleotides 1–1862 of TCF-3 cDNA) detected the 1.9-kb TCF-3 transgene overexpressed in T5, T6, and T10. The 1.2-kb DTD mRNA detected by the DTD S probe (nucleotides 60–436 of DTD cDNA) was down-regulated in T5, T6, and T10. B. Western blot analysis with anti-DTD monoclonal antibody. DTD protein (32 kDa) was down-regulated in T5, T6, and T10. C. Cell proliferation assay with TetraColor One. T5, T6, and T10 showed 8-fold resistance to MMC (IC$_{50}$, 2.4 µg/ml) compared with V (IC$_{50}$, 0.3 µg/ml). D. Multiplex cDNA-PCR for 10 drug resistance-associated genes.](image)
Differential expression analyses of TCF genes in gastric cancer revealed that TCF-1 was expressed at almost the same level in most cases of primary gastric cancer and normal gastric mucosa and that TCF-3 was occasionally up-regulated in primary gastric cancer (Fig. 2B).

MKN28 cells with cytoplasmic β-catenin accumulation and without detectable amounts of TCF-3 mRNA were transfected with TCF-3 expression vector. Three independent MKN28 transformants with TCF-3 overexpression showed about 8-fold resistance to MMC (IC50, 2.4 µg/ml) as compared with MKN28 vector transfectants (IC50, 0.3 µg/ml; Fig. 3C). The IC50 of MMC in MKN28 cells with TCF-3 overexpression is higher than the maximum serum concentration of MMC (1.0 µg/ml) in the patients treated with the usual dose of MMC. Thus, MKN28 transformants with TCF-3 overexpression should be resistant to the usual dose of MMC in vivo.

DTD mRNA was down-regulated in MKN28 transformants with TCF-3 overexpression (Fig. 3A) as well as in primary gastric cancer with TCF-3 up-regulation (data not shown). In addition, the DTD protein was also down-regulated in MKN28 transformants with TCF-3 overexpression (Fig. 3D). TCF-1, endogenously expressed in MKN28 cells, lacks CtBP binding sites, whereas TCF-3 overexpressed in MKN28 transformants contains two CtBP binding sites. The mRNA expression level of TCF-1, LEF-1, and TCF-4, as well as the cytosolic protein level of β-catenin, was not significantly affected by TCF-3 overexpression in MKN28 cells.6 Down-regulation of DTD in MKN28 transformants overexpressing TCF-3 might be due to transcriptional repression of the DTD gene by corepressors associated with overexpressed TCF-3.

DTD is a major two-electron reductase that catalyzes MMC to MMC hydroquinone with potent DNA-alkylating activity (5). Down-regulation of DTD leads to MMC resistance in human gastric and colon cancer cells (6). MMC resistance induced by DTD overexpression is higher than the maximum serum concentration of MMC (1.0 µg/ml) in the patients treated with the usual dose of MMC. Thus, MKN28 transformants with TCF-3 overexpression should be resistant to the usual dose of MMC in vivo.

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References


Unpublished observations.
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