CpG methylation in exon 1 of transcription factor 4 increases with age in normal gastric mucosa and is associated with gene silencing in intestinal-type gastric cancers

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The basic helix-loop-helix (bHLH) family of transcription factors is categorized into distinct classes on the basis of biochemical and functional criteria and each member protein contains an HLH domain composed of two amphipathic helices separated by a loop and a basic DNA-binding domain (1–3). These proteins can form homodimers and heterodimers with other classes of bHLH proteins through the HLH domain to facilitate binding to DNA (4,5). This basic DNA-binding domain is located N-terminal to the HLH domain and makes specific contacts with consensus DNA sequences known as E-boxes (CANNTG) (6). E-box sequences have been found in the promoters of a wide variety of genes, driving their specific activation (7,8). Among the several classes of bHLH families, the class I transcription factors (also called E proteins) are critical regulators in a diverse array of biological processes such as cell growth, differentiation, tissue-specific gene expression and programmed cell death (9–11).

The Transcription factor 4 (TCF4; also known as ITF2, E2-2, ME2 or SEF2) gene product is a member of the class I bHLH family together with the TCF12 (HEB) gene product and the alternatively spliced products E12 and E47 of TCF3 (ITF1, E2A) (9). This gene should not be confused with T-cell transcription factor 4 on human chromosome 10q25.3, which was previously termed TCF4, but is now designated as TCF7L2. Dimerization of TCF4 with other classes of bHLH proteins regulates tissue-specific gene expression through E-box sites and this process, in part, controls differentiation and proliferation in a wide range of cell types such as myocytes (10), osteoblasts (13), B and T lymphocytes (14) and neuronal cells (15). A previous study showed that TCF4 is a downstream target of the WNT/b-catenin/TCF pathway and, like eMYC and cyclin D1, functions as an oncogene when deregulated in human colon cancers (16). In contrast, it has been shown that the enforced expression of TCF4 suppresses the colony-forming efficiency of cells in several cell lines, suggesting that the gene acts as a negative regulator of cell proliferation (11). Very recently, genetic studies demonstrated that loss of one copy of TCF4 causes Pitt-Hopkins syndrome (17–19), a neurodevelopmental disease characterized by mental retardation, seizures and hyperventilation (20–21), suggesting that TCF4 is also critical for human nervous system development.

Epigenetic alterations such as DNA methylation and modification of chromatin structure often occur in neoplasia. It has been firmly established that aberrant methylation of CpG islands in the promoter regions and in the initial exons of many genes occurs in the early stages of carcinogenesis and results in suppressed expression of a variety of genes in a diverse array of cancers (22,23). Many reports have also shown that aberrant methylation of CpG islands leads to inactivation of many genes, particularly in gastric cancers (24–28). Although gastric cancer is the fourth most frequent human cancer and the second leading cause of cancer death in almost every country (29), it is still too often not diagnosed until at an advanced stage. Therefore, identification of effective biomarkers for early stage detection of gastric cancers is an urgent matter.

In this study, we identify TCF4 as a hypermethylated gene in gastric cancers using restriction landmark genomic scanning (RLGS) analysis. We demonstrate prominent hypermethylation of CpG dinucleotides in TCF4 exon 1, which significantly correlates with gene inactivation in early stage gastric cancers and in intestinal-type gastric cancers. Further, the effect of TCF4 on cell growth and migration in gastric cancer cells is investigated.

Materials and methods

Cell lines and tissue samples

Eleven human gastric cancer cell lines, SNU-001, -005, -016, -216, -484, -520, -601, -620, -638, -668, and -719, were obtained from the Korean Cell Line

Introduction

The basic helix-loop-helix (bHLH) family of transcription factors is categorized into distinct classes on the basis of biochemical and functional criteria and each member protein contains an HLH domain.
Methylation-specific polymerase chain reaction

Genomic DNA (1 μg) from cancer cells or clinical samples was modified by sodium bisulfite using the EZ DNA Methylation kit (Zymo Research, Orange, CA) according to the manufacturer’s instructions. Two regions of genomic DNA were targeted for methylation-specific polymerase chain reaction (MSP) analysis: the NotI-linked region in TCF4 exon 8 and the CpG-clustered region in TCF4 exon 1, as shown in Figure 1A. The primer sets were designed with the MethPrimer program (http://www.urogene.org/ methprimer/index.html); for the methylated DNA in TCF4 exon 8, the forward primer 5′-TTATTTTGAAGTTGGAGAAGTGC-3′ and the reverse primer 5′-AAATAA CCTACGCCCCCC-3′ were designed to yield a 198 bp product; for the unmethylated DNA in TCF4 exon 8, the forward primer 5′-TTTGA GAGAAAGTGGCCTA TCCCG-3′ and the reverse primer 5′-AACAGAA AA TAACATAACACCACCC-3′ were designed to yield a 199 bp product; for the unmethylated DNA in TCF4 exon 1, the forward primer 5′-GAATTGTAATTT TCGTGGTCTTC-3′ and the reverse primer 5′-AAAAAAACTCTCCTGGA CACCC-3′ were designed to yield a 258 bp product. MSP reactions were performed with 25 ng bisulfite-modified genomic DNA as follows: 94°C for 5 min, 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 40 s for TCF4 exon 8 or 94°C for 45 s, 59°C for 30 s and 72°C for 60 s for TCF4 exon 1, followed by 72°C for 7 min. PCR products were subjected to 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Bisulfite sequencing

Sequence analysis of bisulfite-modified DNA was performed on 76 Cpg sites covering parts of exon 1 and intron 1 of TCF4, as shown in Figure 1A. Bisulfite-modified DNA (25 ng) was amplified by PCR in a 20 μl reaction containing a forward primer (5′-TTTAGTTTCTCTTTGTTAGTGA-3′) and a reverse primer (5′-AAAAAAATCTCTCTCTTCAAAAAAC-3′) designed to yield a 617 bp product. All amplification reactions were performed as follows: 95°C for 1 min, 40 cycles of 95°C for 45 s, 51°C for 45 s, 72°C for 1 min, followed by 72°C for 5 min. PCR products were cloned into the pGEM-T Easy Vector (Promega), and four to seven clones were randomly chosen for sequencing.

Pyrosequencing

Within TCF4 exon 1, seven Cpg sites were selected for quantitative determination of methylation status (Figure 1A). Bisulfite-modified DNA (100 ng) was amplified by PCR in a 25 μl reaction containing a forward primer (5′-GAGAGGAGAGTTTTGTTAAGAGTTCG-3′) and a biotinylated reverse primer (5′-TACAAAAAACTCTACACCACC-3′) designed to yield a 106 bp product. All amplification reactions were performed as follows: 95°C for 1 min, 50 cycles of 95°C for 30 s, 56°C for 40 s, 72°C for 40 s, followed by 72°C for 5 min. Pyrosequencing was performed according to the manufacturer’s instructions using the sequencing primer 5′-TGGTGTTTTCAGAGATTTG-3′ on the PSQ HS 96A System (Biotage AB, Kungsgatan, Sweden).

5-aza-2′-deoxycytidine and trichostatin A treatment

Gastric cancer cells (SNU-601, -620 and -638) were seeded in 10 cm dishes at a density of 1 × 10^6 cells per dish before treatment. The cells were treated with either 1 μM 5-aza-2′-deoxycytidine (5-aza-dC; Sigma, St Louis, MO) every 24 h for 3 days and then harvested or with 250 nM trichostatin A (TSA; Sigma) for 1 day and then harvested. To test the combined effect of 5-aza-dC and TSA, cells were treated with 1 μM 5-aza-dC every 24 h for 3 days followed by treatment with 250 nM TSA for 1 day. Total RNA was prepared and TCF4 expression was examined by real-time RT–PCR. The average relative mRNA levels were calculated from three independent experiments and from a total of six independent PCR analyses.
Epigenetic silencing of TCF4 in gastric cancer

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer’s protocol with some modifications. Briefly, proteins were cross-linked to DNA by addition of formaldehyde directly to the culture medium and the reaction was quenched with glycine. Cells were then collected and washed twice with ice-cold phosphate-buffered saline containing protease inhibitors and resuspended in sodium dodecyl sulfate lysis buffer. To produce DNA fragments ranging from 200 to 500 bp, lysates were sonicated 21 times for 5 s with 30 s intervals on ice at power setting 3 using a Fisher Sonicator Dismembrator 100 and centrifuged at 13,000 r.p.m. for 10 min at 4°C. The supernatants were diluted in nine volumes of ChIP dilution buffer and precleared with salmon sperm DNA/protein A agarose beads and immunoprecipitated with either 5 μg anti-acetyl-histone H3 antibody (K9, K14) (Upstate Biotechnology, catalog no. 06-599), 5 μg anti-acetyl-histone H4 antibody (K5, K8, K12, K16) (Upstate Biotechnology, catalog no. 06-866) or 5 μg anti-trimethyl-histone H3 (K9) antibody (Upstate Biotechnology, catalog no. 07-442) or with no antibody, according to the manufacturer’s recommendations.

Fig. 1. RLGS analysis of human TCF4 in gastric tumors and gastric cancer cell lines. (A) Schematic diagram of the TCF4 structure at human chromosome 18q21.2 derived from the UCSC Genome Browser (http://genome.ucsc.edu). TCF4 consists of 20 exons (exons 1 and 20 are non-coding) and spans 363 kb. The previously cloned NotI-linked 6B54 sequence (GenBank accession no. CG464927) was found in intron 8. The regions of CpG dinucleotides in exon 1 selected for bisulfite sequencing, MSP, pyrosequencing and MethylLight analysis are indicated below the CpG map. The location of the DNA fragment amplified by the ChIP assay is also indicated. N, Not enzyme site; TSS, transcription start site. (B) Representative examples of decreased spot 6B54 (TCF4 intron 8) intensities from NotI–EcoRV–HinfI RLGS profiles. The uppermost panels show two cell lines in which the intensity of spot 6B54 relative to its neighboring spots was not different from the master profile. The arrow indicates the position of spot 6B54 in each RLGS profile; the upper right panel shows the Master RLGS profile (31). The middle set of panels show gastric cancer cell lines with a decreased intensity of spot 6B54 relative to its neighboring spots. ‘Cells lacking spot 6B54 + normal mucosa’ indicates samples in which DNA from cells lacking spot 6B54 were mixed with DNA from normal mucosa. The lower set of panels show RLGS profiles of gastric tumor tissues compared with those of adjacent normal mucosa tissues from five patients. (C) RT–PCR and methylation-sensitive PCR (MSP) analyses of TCF4. In the upper set of panels, TCF4 mRNA expression was analyzed in the 11 gastric cancer cell lines indicated using RT–PCR. β-Actin was used as an internal control. N, normal mucosa control. In the lower set of panels, TCF4 methylation in exon 1 or intron 8 was analyzed using MSP analysis. M, methylated DNA; U, unmethylated DNA, amplified by specific primers. DW, distilled water used as a negative control.
Immunoepiticipitated DNA was recovered using the QIAquick PCR Purification kit (Qiagen) and amplified in 15 μl reactions containing SYBR Premix EX Taq (Takara) using the primers 5′-TAAAATCCCTTGGCCGTTTG-3′ and 5′-TCAGACCTCATTCTTTGCTT-3′ (located in TCF4 exon 1, exon size 183 bp). Real-time PCR was performed as follows: 95°C for 1 min and 45 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. The amount of immunoepitipated DNA was normalized to the input DNA. The average relative amount of each amplified product was calculated from two independent ChIP experiments and a total of four independent PCR analyses.

Generation of TCF4 expression constructs

Full-length TCF4 cDNA was amplified by PCR from the pBluescriptR-TCF4 plasmid (KUGI clone ID no. HMU003024) provided by Korean UniGene Information [http://kugi.kribb.re.kr/KUGI/index.html] and then cloned into the pGEM-T Easy vector (Promega). The resulting TCF4 cDNA clone was confirmed by sequence analysis and then subcloned into the EcoRI sites of the pEGFP-C1 vector (Clontech). The expression of green fluorescence protein (GFP)-TCF4 from the resulting pEGFP-C1-TCF4 plasmid was confirmed by western blotting using an anti-GFP antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Colony-forming assay

To analyze anchorage-dependent growth, a colony formation assay was performed on human colon cancer cell lines. TCF4 expression was confirmed by sequence analysis and then subcloned into the pEGFP-C1 vector (Clontech). The expression of green fluorescence protein (GFP)-TCF4 from the resulting pEGFP-C1-TCF4 plasmid was confirmed by western blotting using an anti-GFP antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Cpf1 methylation and histone acetylation regulate transcriptional silencing of TCF4 in gastric cancer cells

Bisulfite sequencing in exon 1 and intron 1 revealed extensive hypermethylation (75–98%) in the eight cell lines with little or no TCF4 expression, whereas hypomethylation (0.8–18%) was detected in the three TCF4-expressing cell lines (Figure 2A). Pyrosequencing analysis of seven CpG dinucleotides within exon 1 of TCF4 was used to confirm the bisulfite sequencing results (Figure 2A, right panel). The methylation status of the seven CpG sites examined by pyrosequencing analysis was representative of the 76 CpG sites examined by bisulfite sequencing. Real-time RT–PCR analysis also revealed that silencing of TCF4 expression correlated with the methylation status of exon 1 determined by pyrosequencing (Figure 2B), suggesting that Cpf1 methylation at exon 1 is associated with the inactivation of TCF4 in human gastric cancer cell lines. We next determined that TCF4 mRNA expression was restored in SNU-601, -620 and -638 cells after 5-aza-dC and/or TSA treatment (Figure 2C). This result suggests that TCF4 mRNA expression in gastric cancer cells may be regulated by both Cpf1 methylation and histone acetylation. We therefore examined histone acetylation and methylation in chromatin associated with TCF4 exon 1 using ChIP analysis. Histone-associated DNA fragments immunoprecipitated with antibodies against acetyl-H3 (acetylated at residues K9 and K14), acetyl-H4 (acetylated at residues K5, K8, K12 and K16) or H3K9me3 (acetylated at residue K9 and methylated at residue 3) were subjected to PCR analysis using primers designed to amplify a region of TCF4 exon 1. The acetylation of histones H3 and H4 within TCF4 exon 1 was elevated in SNU-484 and SNU-601 cells, in which TCF4 is unmethylated and transcriptionally active, compared with SNU-601 or -638 cells, in which TCF4 is hypermethylated and transcriptionally silent (Figure 2D). In contrast, anti-H3K9me3 immunoprecipitation enriched amplification of TCF4 exon 1 in SNU-601 and -638 cells (Figure 2D). These results clearly indicate that histone modification is also likely involved in transcriptional silencing of TCF4 in gastric cancers.

The status of TCF4 expression in primary gastric tumors

To test whether Cpf1 methylation in TCF4 exon 1 is abnormally increased in primary tumors compared with the corresponding normal tissue, we next performed MSP analysis on clinical samples. Figure 3A shows that a single PCR product was amplified using primers for unmethylated Cpf1 sequence from all gastric tumors, as well as from normal tissues, suggesting the presence of normal mucosa or stromal cells in the gastric tumor specimens that may have contributed to the amplification of unmethylated products. Nonetheless, a prominent PCR product for methylated Cpf1 sequence was detected in all tumors except for 2 samples (18 and 410) of 20 tumors, whereas no or weak bands were observed in 16 of the corresponding normal tissue.
specimens. This result suggests that CpG methylation in TCF4 exon 1 may be a feature of gastric tumors.

To elucidate the status of TCF4 expression during gastric tissue carcinogenesis, we investigated TCF4 mRNA expression in 77 paired clinical tissues using real-time quantitative RT–PCR and examined the associated clinicopathologic parameters. TCF4 mRNA expression in tumor tissues was significantly lower than in adjacent normal tissues ($P < 0.0001$) after normalization to $\beta$-actin mRNA levels (Figure 3B). Loss of expression (LOE) was assigned to tumors in which the expression of TCF4 mRNA was less than or equal to half of that in adjacent normal tissues. TCF4 LOE was detected in 38% (29 of 77) of primary tumors.

Quantitative methyl-CpG analysis of the same seven CpG sites in TCF4 exon 1 described in Figure 1A was performed on the 77 paired clinical tissues via pyrosequencing. The mean percent methylation was 10.9, 13.8, 13.5, 15.1, 12.4, 13.3 and 13.3% for each of the seven CpG sites examined in TCF4 exon 1 from normal tissues. The overall mean percent methylation was calculated from the average of these individual values and was determined to be 13.2. In the same manner, the overall mean percent methylation in TCF4 exon 1 from tumor tissues was 34.7%, revealing a significant difference compared with normal tissues ($P < 0.0001$) (Figure 3C). As shown in Table I, the mean percent methylation was significantly higher in tumors with TCF4 LOE (43.0 ± 23.2%) than in those with TCF4 expression comparable with their matched normal tissue (29.8 ± 19.1%) ($P = 0.006$), indicating that increased CpG methylation correlated with decreased TCF4 expression in primary gastric tumors. Clinicopathologic characteristics of patients with respect to tumor TCF4 expression levels are also shown in Table I. TCF4 LOE was more frequent among females (14/26, 53.8%) than males (15/51, 29.4%) ($P = 0.04$). In particular, TCF4 LOE was significantly more common in intestinal-type than diffuse-type gastric cancers ($P = 0.0004$) and in early gastric cancers than in advanced gastric cancers ($P = 0.004$), suggesting that TCF4 LOE may be an early event in the multistep process leading to gastric carcinogenesis.

Fig. 2. TCF4 CpG methylation and histone modifications correlate with TCF4 silencing in gastric cancer cell lines. (A) Quantitative measurement of CpG dinucleotide methylation in TCF4 exon 1. In the upper panels, each horizontal row of boxes represents a single clone analyzed by bisulfite sequencing. Each small square box represents a CpG site. Filled and open square boxes indicate methylated and unmethylated CpG sites, respectively. The bar with an asterisk indicates the seven CpG sites used for pyrosequencing analysis. Representative pyrograms for SNU-001 and SNU-668 cells are shown in the lower set of panels. Numerical values at right represent the average percent methylation of the indicated CpG sites determined by bisulfite sequencing (bisulfite) or by pyrosequencing (pyro). (B) Relative TCF4 expression in gastric cancer cell lines. Relative TCF4 expression was obtained using real-time RT–PCR, and results from three independent analyses are expressed relative to $\beta$-actin mRNA levels. (C) Restoration of TCF4 expression in gastric cancer cells after treatment with the indicated drugs. Three gastric cancer cell lines, SNU-601, SNU-620 and SNU-638, were treated with 5-aza-dC, TSA or both. For each cell line, TCF4 expression was measured using real-time RT–PCR and results from three independent analyses are expressed relative to $\beta$-actin mRNA levels. (D) ChIP assays of the TCF4 exon 1. Chromatin DNA was immunoprecipitated with antibodies specific for acetyl-H3 (AcH3), acetyl-H4 (AcH4) or trimethyl-H3-K9 (H3K9me3). DNA fragments corresponding to 183 bp in the TCF4 exon 1 (see Figure 1A) were amplified by PCR. The amount of immunoprecipitated DNA was normalized to the input DNA.
Age-related TCF4 CpG methylation in gastric tumors and adjacent normal mucosa

No mean age differences were found between patients with tumors having TCF4 LOE and those having non-LOE tumors. When patient ages were subdivided into four age groups, however, the mean percent TCF4 methylation determined by pyrosequencing of the seven CpG sites in exon 1 in age group 1 (≤50 years, n = 17) was ~1.7 and 24.5% in gastric mucosa and tumors, respectively. The mean percent TCF4 methylation in gastric mucosa and tumors was 9.5 and 30.9%, respectively, in age group 2 (51–60 years, n = 22), 18.2 and 41.0%, respectively, in age group 3 (61–70 years, n = 26) and 25.3 and 42.5%, respectively, in age group 4 (>70 years, n = 12), as shown.
TCF4 mRNA levels could not be detected in normal tissues of older patients because these tissues do not contain the extensively hypermethylated clone.

**TCF4 CpG methylation during gastric carcinogenesis**

To elucidate the methylation status of TCF4 during gastric carcinogenesis, we performed MethyLight analysis on the CpG sites in exon 1 (shown in Figure 1A) on 35 sets of paraffin-embedded tissues of chronic gastritis, intestinal metaplasia, gastric adenomas and gastric carcinomas. No CpG methylation in TCF4 exon 1 was found in the chronic gastritis samples but a gradual increase in CpG methylation from intestinal metaplasia (mean percent of methylated reference 0.5%, range of 0.0–11.5%) to gastric adenomas (mean 2.4%, range of 0.0–31.8%) or gastric carcinomas (mean 3.3%, range of 0.0–49.7%). This result suggests that CpG methylation in TCF4 exon 1 is initiated at an early stage such as intestinal metaplasia during gastric carcinogenesis and tends to accumulate along the multistep carcinogenesis.

**In vitro effects of TCF4 on cell growth and migration in gastric cancer cells**

To determine whether TCF4 is involved in cell growth in gastric cancer cells, we introduced a TCF4 expression vector into SNU-638 cells, where methyl-CpG-associated endogenous TCF4 silencing was demonstrated (see Figure 2). TCF4 expression in cells transfected with the pEGFP-C1-TCF4 expression plasmid, but not in the parental SNU-638 cells or in cells transfected with the control pEGFP-C1 vector, was confirmed by western blotting using a monoclonal anti-GFP antibody (Figure 4A). In the monolayer assay used to evaluate anchorage-dependent growth, the number of colonies from pEGFP-C1-TCF4 transfectedants was much smaller than that of control pEGFP-C1 transfectedants (P < 0.0499) (Figure 4B). A soft agar assay for anchorage-independent growth also showed decreased colony size in pEGFP-C1-TCF4 transfectedants compared with control pEGFP-C1 transfectedants (P < 0.0001) (Figure 4C). We also addressed whether transfection with pEGFP-C1-TCF4 resulted in any morphological alterations but did not note any morphological change or apoptosis at 24 h after transfection. We further examined whether reducing TCF4 expression by RNA interference altered cell migration in gastric cancer cells. RT–PCR analysis of SNU-484 and SNU-668 cells transfected with a broad range of TCF4-siRNA concentrations indicated that 60 nM TCF4-siRNA reduced TCF4 transcript levels in both cell lines compared with mock-transfected controls (Figure 4D). Transwell assays also demonstrated increased cell migration in SNU-484 (P < 0.0037) and SNU-668 cells (P < 0.0002) transfected with TCF4-siRNA compared with mock-transfected cells (Figure 4E). These results suggest that TCF4 silencing may be associated with cell growth and migration in gastric cancer cells.

**Discussion**

In this study, we identified epigenetic silencing of TCF4 in human gastric cancers and demonstrated that this process is linked to cell growth and migration. Genetic and epigenetic alterations in tumor suppressor genes are a prerequisite of cancer development and progression. In particular, epigenetic alterations within tumors are inevitable and often promote further genetic modifications (35). Similar to the findings in this study, CpG methylation of promoter regions or the first exon of tumor suppressor genes has been established as an important mechanism for gene silencing (36). Moreover, silencing of TCF4 was relieved in several gastric cancer cell lines upon treatment with a DNA methyltransferase and/or a histone deacetylase inhibitor. Thus, our data demonstrate epigenetic regulation of TCF4 in gastric cancers.

Although our understanding of the molecular mechanisms of the pathology of sporadic gastric cancers is increasing, the cellular events that trigger initiation of carcinogenesis in human gastric mucosa cells remain unclear (37). It is widely accepted, however, that epigenetic

### Table I. Relative TCF4 mRNA expression in tumors with respect to clinicopathologic characteristics

| Clinicopathologic parameter | Gastric tumors with decreased relative TCF4 expression \( \geq 50\% \) | \( < 50\% \) decrease in TCF4 expression | \( P \) value \( b \) |
|-----------------------------|---------------------------------|---------------------------------|-----------------|
| Mean patient age (in years \( \pm SD \)) | 61.1 ± 11.4 | 58.9 ± 11.5 | 0.41 |
| Gender | | | |
| Male | 15 | 36 | 0.04 |
| Female | 14 | 12 | |
| Tumor size in cm\(^2\) (mean ± SD) | 5.2 ± 2.4 | 5.7 ± 2.3 | 0.39 |
| Histology | | | |
| Intestinal | 22 | 15 | 0.0004 |
| Diffuse | 7 | 30 | |
| Mixed\(^a\) | 0 | 3 | |
| Tumor progression\(^c\) | EGC | 12 | 6 | 0.004 |
| AGC | 17 | 42 | |
| Lymph node metastasis | Negative | 14 | 14 | 0.09 |
| Positive | 15 | 34 | 0.0499 |
| TCF4 methylation\(^d\) (mean ± SD) | 43.0 ± 23.2 | 29.8 ± 19.1 | 0.006 |

\(^a\) All tumors were classified into two subtypes: those in which the decrease in TCF4 mRNA expression was 50% or more compared with adjacent normal tissue (≥50% decrease) and those in which the decrease in TCF4 mRNA expression was <50% compared with adjacent normal tissue (<50% decrease).

\(^b\) Analyzed by Student’s \( t\)-test or by \( \chi^2 \) test.

\(^c\) Size was calculated based on the largest diameter measured for each tumor.

\(^d\) Mixed type tumors were excluded from this analysis because of a small sample number (n = 3).

\(^e\) Partial, early gastric cancer; AGC, advanced gastric cancer.

\(^f\) The percent methylation at seven distinct CpG sites in TCF4 exon 1 was determined by pyrosequencing analysis. The percentages were then averaged to calculate TCF4 methylation.

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alterations are a prerequisite of virtually all tumors and that epigenetic alterations facilitate the accumulation of further genetic modifications that result in cancer progression through clonal expansion of cells with a resulting proliferative advantage (35). In this study, we demonstrated that 38% of the gastric tumors examined displayed transcriptional factor family, and that TCF3 acts as a tumor suppressor (39). In this study, examination of clinicopathologic characteristics of tumors showed that TCF4 silencing was significantly more prevalent in intestinal type and in early stage gastric cancers than in diffuse type or in advanced stage gastric cancers. Recent results have shown that promoter hypermethylation in many genes occurs in the non-cancerous tissues adjacent to gastric cancer tumors (40,41) and in non-neoplastic gastric mucosa of patients without gastric cancer (42), indicating that an overall deregulation of the DNA methylation machinery may be an early event in the formation of gastric cancers and may become more severe as carcinosgenesis progresses.

Because the normal gastric mucosa and tumor tissues used in this study were not dissected by a technique such as laser-captured microdissection, the normal gastric mucosa may have actually been normal-appearing precancerous gastric mucosa, and likewise, the tumor tissues may have included some normal mucosa or precancerous cells. Although our samples may have contained these possible mixtures, our data showed that CpG methylation in TCF4 exon 1 gradually increased in an age-dependent manner in all normal-appearing gastric mucosa samples examined—the average percent of methylated CpG sites ranging from 1.7% in age group 1 (<50 years) to 25.3% in age group 4 (over 70 years). These data are highly relevant to gastric neoplasia because the incidence of sporadic gastric tumors is strongly age related (43). In agreement with this, it is worthwhile to note that the mean percent TCF4 CpG methylation in normal-appearing gastric mucosa of older patients (25.3% for patients over age 70) was similar to that in gastric tumor tissues from younger patients (24.5% for patients younger than age 50), suggesting that gastric mucosa from patients over age 70 may be predisposed to neoplasia. However, it is unlikely that TCF4 CpG methylation contributes to decreased TCF4 expression in normal gastric mucosa in older patients based on our observation that normal gastric mucosa from older patients contains no extensively hypermethylated clone, which may be correlated with TCF4 expression, in contrast to its presence in gastric tumors from younger patients and in somatic gastric cancer cell lines. In fact, no significant difference in TCF4 expression in the normal mucosa was detected between age groups. Although CpG methylation in TCF4 exon 1 was not found in chronic gastritis, a gradual increase in CpG methylation from intestinal metaplasia to neoplastic gastric mucosa, such as gastric adenomas or gastric carcinomas, was noted. Therefore, it is reasonable to propose that increased TCF4 CpG methylation in normal-appearing gastric mucosa may be due to local methylation of CpG sites initiated at an early stage during gastric carcinogenesis (such as in intestinal metaplasia), rather than due to extensive CpG methylation as proposed in the ‘field cancerization effect’ in an epithelial carcinogenesis model in which the development of a field of genetically altered cells plays a central role (44).

TCF4 consists of 20 exons (exons 1 and 20 are non-coding), spans 363 kb, and encodes at least two isoforms of the TCF4 protein that differ with respect to the presence of four amino acids (RSRS) located 17 residues N-terminal to the HLH domain (19). Although previous studies have presented controversial cellular roles for TCF4 (11,16), our data show that overexpression of TCF4 reduced colony formation in an anchorage-dependent and -independent manner, supporting a role for TCF4 as a negative regulator of cell proliferation (16). Because it has also been shown that the functional patterns of TCF4 are very similar to those of TCF3, another member of the bHLH transcriptional factor family, and that TCF3 acts as a tumor suppressor in several cancer cell lines (11), TCF4 may be associated with the induction of apoptosis. Furthermore, no significant correlation between β-catenin mutations and expression of TCF4 in hepatoblastomas has been demonstrated (45), although β-catenin-binding.
proteins, such as Icat or Chibby, may regulate β-catenin function and its ability to activate TCF7L2-regulated transcription (46,47). A possible explanation for the observed variation in TCF4 expression is that the expression may be controlled by other signaling pathways in a cell type-specific manner. We showed that knock down of TCF4 using TCF4-siRNA significantly increased cell migration in vitro, also suggesting an inhibitory effect of TCF4 on cell migration. Thus, our results suggest that TCF4 has a possible negative effect on both cell growth and migration in gastric cancer cells. In this study, however, because no correlation was found between lymph node metastasis and TCF4 silencing in clinical samples and because we found that TCF4 silencing was significantly more frequent in early stage gastric cancers than in advanced stage gastric cancers, further analysis is needed to resolve the role of TCF4 silencing in carcinogenesis. However, CpG methylation of TCF4 may prove to be a useful molecular biomarker for assessing the risk of gastric cancer development and drug therapies targeting TCF4 expression in gastric cancers may improve prognosis for gastric cancer patients.

Supplementary material
Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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