TCF12 Protein Functions as Transcriptional Repressor of E-cadherin, and Its Overexpression Is Correlated with Metastasis of Colorectal Cancer*§

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A correlation of TCF12 mRNA overexpression with colorectal cancer (CRC) metastasis was suggested by microarray data and validated by the survey of 120 patients. Thirty-three (27.5%) of the 120 patients showed tumor TCF12 mRNA overexpression and had a higher rate of metastatic occurrence (p = 0.020) and a poorer survival outcome (p = 0.014). Abundant TCF12 levels were also observed in human CRC cell lines such as SW620 and LoVo, but a relatively low level was detected in SW480 cells. Knockdown of TCF12 expression in SW620 and LoVo cells drastically reduced their activities of migration, invasion, and metastasis. Tight cell-cell contact and an increase in E-cadherin but a concomitant decrease in fibronectin were observed in TCF12-knockdown cells. Connexin 26, connexin 43, and gap-junction activity were also increased upon TCF12-knockdown. In contrast, ectopic TCF12 overexpression in SW480 cells facilitated fibronectin expression and cell migration and invasion activities but diminished cellular levels of E-cadherin, connexin 26, connexin 43, and gap junction. A physical association of TCF12 with the E-cadherin promoter was evidenced by chromatin immunoprecipitation assay. TCF12 was tightly correlated with cellular expression of Bmi1 and EZH2 and was co-immunoprecipitable with Bmi1 and EZH2, suggesting that TCF12 transcriptionally suppressed E-cadherin expression via polycomb group-repressive complexes. Clinically, TCF12 mRNA overexpression was also correlated with E-cadherin mRNA down-regulation in the tumor tissues of our 120 patients (p = 0.013). These studies suggested that TCF12 functioned as a transcriptional repressor of E-cadherin and its overexpression was significantly correlated with the occurrence of CRC metastasis.

Metastasis is the major cause of colorectal cancer (CRC) mortality. Identification of metastasis-associated genes is essential for understanding the underlying mechanisms, developing new markers for early detection, and ultimately discovering new therapeutic targets. Development of new techniques in proteomic analysis has greatly helped identify molecules involved in the pathogenesis of metastasis (1–3). The advent of microarray technologies has also enhanced the search for genetic factors related to metastasis (4–6). To identify novel metastasis-associated gene candidates in CRC, this study used microarray technology to determine the gene expression profiles of four CRC patients (two diagnosed with tumors confined in the primary sites and two diagnosed with metastasis). Elevated mRNA expression of the TCF12 gene was observed in the tumor tissues of both patients with metastasis.

The product of TCF12 gene (TCF12, also called HTF4 or HEB) is a member of helix-loop-helix (HLH) protein family (7, 8). HLH proteins have been divided into seven classes according to their tissue distribution, dimerization abilities, and DNA binding specificities (9). The class I proteins, encoded by TCF3 (E12, E47), TCF4 (E2-2), and TCF12 (HTF4) genes, are also known as E proteins because of their direct DNA (E-box) binding ability. These proteins are widely expressed in many tissues and can form homodimers or heterodimers. The class II proteins, including MyoD, myogenin, and TWIST, can form heterodimers with class I members and show a tissue-specific expression pattern. The class III proteins contain a leucine zipper domain adjacent to the HLH domain and include the Myc family of transcription factors. The members of class IV define a family of proteins such as Mad, Max and Mxi, which can dimerize with Myc. Class V proteins lack the basic region and thus cannot bind DNA directly. The member Id acts as class I and class II dominant-negative factors. Class VI members contain a proline in their basic region, and class VII proteins are categorized by their HLH-PAS domain and include members such as hypoxia-inducible factor 1α and aromatic hydrocarbon receptor.

E-cadherin is a cell-cell junction protein (10). Down-regulation of E-cadherin is a hallmark of epithelial-mesenchymal transition (EMT), an important process in embryonic development (11). During the malignant progression of solid tumors,
the changes in cell adhesion and migration are similar to those occurring during EMT and E-cadherin is down-regulated. Loss or reduction of E-cadherin in tumor cells can arise from gene mutation, chromosomal deletion, proteolytic cleavage, gene silencing, and transcriptional repression (12). Certain transcription factors, including members of Snail, ZEB, and HLH families, are able to repress E-cadherin expression and play important roles in tumor progression (11). In HLH proteins, the class II member TWIST1 is a well characterized E-cadherin repressor and EMT inducer (13), and it was reportedly associated with Bmi1 for its function (14). Bmi1 and EZH2 are the important components of polycomb group-repressive complex 1 (PRC1) and PRC2, respectively (15). PRC2 is a transcriptional repression initiation complex, and its binding to E-cadherin promoter region allows EZH2 to methylate the lysine-27 of histone H3. PRC1 furthermore recognizes trimethylated histone H3 and maintains the repression of E-cadherin (15). TWIST1 is associated with malignancy in animal studies (13, 16), and its correlation with tumor metastasis and poor prognosis has also been reported in human cancers (17–21). Recently, the HLH class I member TCF12 was shown to suppress E-cadherin expression during the early stages of renal tubular epithelial cell dedifferentiation (22). Whether or not TCF12 plays a role in cancer development and progression has not been reported yet, and its expression status in clinical specimens also requires to be investigated.

In this study tumor TCF12 mRNA overexpression was detected in 33 (27.5%) of 120 CRC patients and was suggested as a risk factor for the occurrence of CRC metastasis. TCF12-knockdown or ectopic overexpression in CRC cell lines indicated that TCF12 facilitated fibronectin expression but reduced E-cadherin, connexin 26, connexin 43, and gap-junction function, which were accompanied by the enhancement of CRC cell migration, invasion, and metastasis. TCF12 was evidenced by chromatin immunoprecipitation (ChIP) assay to be associated with the promoter region of E-cadherin gene and was co-expressed and co-immunoprecipitable with Bmi1 and EZH2, suggesting that TCF12 transcriptionally repressed E-cadherin expression via PRC1 and PRC2 participation. The clinical correlation of tumor TCF12 mRNA overexpression with tumor E-cadherin mRNA down-regulation was also observed in the 120 CRC patients. These studies suggested that TCF12 was a transcriptional repressor of E-cadherin and facilitated the occurrence of CRC metastasis.

**EXPERIMENTAL PROCEDURES**

**Clinical Specimens**—Tumor tissue specimens were obtained from 140 patients who underwent surgical resection at Taipei Veterans General Hospital for CRC between 1993 and 2004. Written informed consent from each patient was obtained in accordance with the requirement of the Medical Ethics Committee of Taipei Veterans General Hospital. The adjacent non-tumor tissues were obtained from the distal edge of each resection at least 10 cm away from the tumor and were pathologically certified to be free from tumor cells. The tissue specimens were frozen in liquid nitrogen immediately after resection and PBS rinse, and kept at -80 °C until RNA or/and protein extraction. The clinical stage was estimated from each patient from surgical and pathological reports using the tumor-node-metastasis (TNM) staging system. Patients with tumors that could not be completely removed, such as carcinomatosis or multiple metastases over bilateral liver lobes, and patients who had received any chemo- and/or radiotherapeutic treatment before surgery were excluded from this study.

**Cell Lines**—The human CRC cell lines SW620, SW480, HCT-8, and HCT-116 were cultivated at 37 °C in an atmosphere of 95% air and 5% CO2 with RPMI medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 20 mM L-glutamine. The human CRC cell line LoVo was cultivated using the same conditions but Ham’s F-12 medium supplemented with 20% FBS was used instead.

**Cloning of TCF12 Knockdown and TCF12-overexpressing Cells**—To generate TCF12-knockdown cells, SW620 or LoVo cells were transfected with the TCF12 shRNA-expressing plasmid (2 μg of DNA/105 cells for 48 h) using Effectene transfection reagent (Qiagen, Valencia, CA). The transfectants were selected against 2 μg/ml puromycin for 10 days, and cell clones were screened for TCF12 down-regulation by RT-PCR and immunoblot analysis. The empty vector pLKO.1 puro and four plasmids expressing four different 21-mer shRNA sequences directed against TCF12 mRNA were obtained from the National RNAi Core Facility (Taipei, Taiwan). To generate TCF12-overexpressing cells, SW480 cells were transfected with pcDNA3-HA plasmid carrying TCF12 cDNA (2 μg of plasmid DNA per 105 cells for 48 h) using Effectene reagent. After selection against 500 μg/ml G418 for 2 weeks, cell clones were screened for TCF12 overexpression by RT-PCR and immunoblot analysis.

**Mouse Model**—Vector-transfectant or TCF12-knockdown SW620 cells were injected through the tail vein into 7-week-old non-obese diabetic-severe combined immunodeficiency (NOD-SCID) mice using 27-gauge needles (2 × 105 cells in 200 μl PBS for each mouse). Mice were sacrificed after 2 weeks, and their lungs were excised and fixed overnight with 10% formalin. The metastatic tumor nodules on the surface of the lungs were examined, counted, and photographed under a Leica MZ6 dissecting microscope (Wetzlar, Germany). The nodules were confirmed to be tumor tissues by histopathological examination after tissue section and hematoxylin and eosin staining.

**RNA Isolation**—Each tissue sample with 2 mg of tissue pieces was added to 1 ml of Trizol reagent (Invitrogen) in a homogenizer tube containing ceramic beads (Roche Applied Science). Homogenization was performed in a MagNA Lyser (Roche Applied Science) by 5 cycles of oscillation at 6500 rpm for 20 s and chilling on ice for 1 min. Tissue lysates were further subjected to RNA isolation using the RNasy protocol (Qiagen). Purified RNA was quantified by measuring the optical density at 260 nm with the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The RNA integrity was measured using a Bioanalyzer 2100 with the RNA 6000 Nano LabChip kit (Agilent Technologies, Santa Clara, CA). For RNA isolation from cultured cells, cells were suspended in Trizol reagent for lysis without ceramic beads-mediated homogenization.
Gene Expression Array Analysis—Aliquots of eight RNA samples (0.5 μg for each) selected from the tumor tissues and the corresponding non-tumor tissues of two patients with metastasis and two patients without metastasis were amplified by Low RNA Input Fluor Linear Amp kit (Agilent) and labeled with Cy3- or Cy5-dUTP (GE Healthcare). RNA derived from tumor parts was labeled with Cy5, and RNA from the corresponding non-tumor parts was labeled with Cy3. Both Cy-labeled cRNAs were fragmented to about 50–100 nucleotides and then pooled and hybridized to Agilent Whole Human Genome Oligo Microarray 44K at 60 °C for 17 h. After washing and drying by nitrogen blowing, microarrays were scanned with an Agilent microarray scanner at 535 nm for Cy3 and 625 nm for Cy5. The scanned images were analyzed by Feature Extraction 9.1 software (Agilent) to quantify the signal and background intensity for each feature, and normalization of the data were performed using the rank-consistency-filtering LOWESS method. Only the features with a signal-to-noise ratio >2.6 in either the Cy3 or Cy5 channel were retrieved for further analysis. Microarray data are available through GEO (accession no. GSE24639).

Polymerase Chain Reaction (PCR)—One microgram of RNA from each sample was subjected to reverse transcription (RT) using SuperScript II Reverse Transcriptase (Invitrogen). During cDNA synthesis, random primers were used to replace oligo-dT primers. After RT, the synthesized cDNA was used as the template for subsequent PCR reactions. The sequences of forward and reverse primers, the PCR conditions, and the lengths of the PCR products, respectively, were as follows: TCF12 (forward, 5'-AAC-AAC-GCA-TGG-CCG-CTA-3'; reverse, 5'-GGG-ATC-AAG-GGT-CGG-AGT-3'; 94 °C (30 s), 65 °C (1 min) and 72 °C (1 min) for 40 cycles; 220 bp); Bmi1 (forward: 5'-CGC-GAG-GAG-AGC-TTG-TCA-TTG-3'; reverse, 5'-CGC-GCC-GCG-CCT-CTT-TCT-3'; 94 °C (1 min), 59 °C (1 min), and 72 °C (1 min) for 30 cycles; 734 bp); fibronectin (forward, 5'-ACC-AAC-CTA-CGG-ATG-ACT-3'; reverse, 5'-GCT-CAT-CAT-CTG-GCC-ATT-CTT-3'; 94 °C (30 s), 56 °C (30 s), and 72 °C (1 min) for 30 cycles, 229 bp); connexin-3 (forward, 5'-GGG-ATC-AAG-GGT-CGG-AGT-3'; reverse, 5'-GAA-GGT-GAA-GGT-CGG-AGT-3'; 94 °C (30 s), 56 °C (30 s), and 72 °C (1 min) for 30 cycles, 229 bp); 3'-GAPDH (forward, 5'-GGC-GAA-GAT-GGT-GAG-3'; reverse, 5'-GGA-GGT-GAA-GGT-CGG-3'; 94 °C (30 s), 56 °C (30 s), and 72 °C (1 min) for 30 cycles, 220 bp). All PCR reactions were started at 94 °C for 5 min and terminated at 72 °C for 7 min. Real-time quantitative PCR (qPCR) was performed in a RotorGene 3000 system (Corbett Research, Mortlake, Australia) using SYBR Green PCR Master Mix (Cambrex Co., East Rutherford, NJ). Data were analyzed using RotorGene software Version 5.0 (Corbett Research). GAPDH levels were used as internal controls for normalization. The difference in the TCF12 expression levels of the tumor and adjacent non-tumor tissue was considered as overexpression or down-regulation when the ratio was >2 and <0.5, respectively.

Immunoblot Analysis—Cells were lysed in 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.3% SDS, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Aliquots (40 μg each) of cell lysates were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked in PBST (PBS containing 0.1% Tween 20) plus 5% non-fat milk for 60 min at room temperature and then incubated overnight at 4 °C with anti-TCF12 or anti-GAPDH antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) in PBST plus 5% nonfat milk. The membranes were washed 3 times for 15 min each with PBST at room temperature and then incubated for 60 min with horseradish peroxidase-conjugated secondary antibody. After three washes with PBST, immunoreactive bands were detected by enhanced chemiluminescence (Amersham Biosciences).

Nuclear Extraction and Immunoprecipitation—Cells were lysed in lysis buffer consisting of 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, 2 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A. The lystate was gently layered above the lysis buffer plus 30% (w/v) sucrose and centrifuged at 1700 × g for 10 min at 4 °C. The pellet fraction was saved and treated for 30 min at 4 °C with radioimmune precipitation assay buffer consisting of 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM dithiothreitol, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A. After centrifugation at 20,000 × g for 15 min, the supernatant was saved as nuclear extract. For immunoprecipitation, each nuclear extract sample (500 μg in 500 μl of radioimmune precipitation assay buffer) was incubated overnight at 4 °C with 10 μg of anti-TCF12 monoclonal antibody (Santa Cruz Biotechnology). After the addition of protein A/G-Sepharose and incubation for another 2 h, the immunoprecipitate was washed 5 times with radioimmune precipitation assay buffer. The immunoprecipitate was resolved by 12% SDS-PAGE and analyzed by immunoblotting with anti-Bmi1 (Millipore, Billerica, MA) or anti-EZH2 (Cell Signaling Technology, Beverly, MA) antibody.

Cancer Cell Migration and Invasion Assays—For migration assays, cells were seeded in 6-well plates and grown to confluence. The monolayer of cells was wounded with a white tip, washed twice with PBS, and incubated at 37 °C for 16 h. Pictures of the cells migrating into the wounded area were taken every 10 min and quantified using Image-Pro Plus Version 5.0.2 software (MediaCybernetics Inc., Silver Spring, MD). To assay cellular invasion, Transwell inserts (8-μm pores) were first coated with Matrigel (1:5 diluted with RPMI medium) and incubated at 37 °C for 30 min. Cells were suspended in 0.5% serum-containing RPMI medium and inoculated in the top chambers of Transwell inserts. Cells were allowed to migrate for 16 h through the Matrigel toward the bottom chambers containing RPMI medium plus 10% FBS. The filters of Transwell inserts...
were then fixed and stained with Giemsa, and pictures were taken of the invasive cells on the filters and counted by Image-Pro Plus software.

**ChIP Assay**—A ChIP assay was performed according to the instruction manual of the EZ-ChIP kit (Millipore). Briefly, cells were treated with 1% formaldehyde to cross-link proteins to DNA before cell lysis in SDS buffer and DNA fragmentation by sonication. Cell lysates were then precleared using protein G-conjugated agarose, 10-μl aliquots of cell lysates were saved as “input” fractions, and the remaining lysates were immunoprecipitated with mouse preimmune immunoglobulin (IgG) or anti-TCF12 antibody. Finally, the DNA from the immunoprecipitates was extracted and subjected to PCR amplification. The E-box-containing E1 (−480 to −280) and E2 (−172 to +30) regions of E-cadherin promoter were amplified by 40 cycles at 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s. The primer sequences were: E1 (forward, 5′-TGG-TGG-TGT-GCA-CCT-GTA-CT-3′; reverse, 5′-GGG-CTT-TTA-CAC-CTT-GCT-GA-3′) and E2 (forward, 5′-TAG-AGG-GTC-ACC-GGG-TCT-AT-3′; reverse, 5′-TCA-CAG-GTG-CTT-TGC-AGT-TC-3′).

**Gap-junction Activity Assay**—The transfer of calcein between cells was analyzed to evaluate gap-junction activity (23). To prepare dye-donor cells, cells were trypsinized, resuspended in 0.3M glucose, and incubated for 30 min in a solution of 50 nM calcein AM (calcein acetoxymethyl ester) and 90 nM DiI (Invitrogen). After washing three times with PBS, the dye-donor cells were added to a monolayer of steals with the same type (i.e. dye recipient cells) at a ratio of 1:10 (donor/recipient). Donor and recipient cells were cocultured for 3 h, then harvested by trypsinization, resuspended in PBS, and immediately subjected to analysis using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences).

**Statistical Analyses**—Results of cell culture experiments were obtained from at least three independent experiments, and differences of the data were considered significant if \( p < 0.05 \) by performing Student’s \( t \) test. Differences in the patient ages, tumor sizes, and serum levels of carcinoembryonic antigen (CEA) and cancer antigen CA19-9 among the patient groups with different TCF12 expression status were evaluated by one-way analysis of variance. The Pearson \( \chi^2 \) test was used to analyze the relationships of the TCF12 expression status with patient clinicopathological characteristics. Univariate and multivariate logistic regression analyses were performed to determine whether tumor TCF12 mRNA overexpression could be a risk factor for CRC metastasis. Patient overall survival rates were calculated by Kaplan-Meier curves. The prognostic significance of tumor TCF12 mRNA overexpression was evaluated using Cox regression analysis. Results were considered significant if the \( p \) value was \(< 0.05\) (two-tailed test).

**RESULTS**

**TCF12 Is Overexpressed in CRC**—Two patients diagnosed with non-metastatic CRC and two patients diagnosed with metastasis from CRC were randomly selected for the preliminary study. Differential gene expression in the tumor tissue and adjacent non-tumor tissue from each patient was initially assessed through gene expression microarray analysis. The differential gene expression profiles comparing metastatic and non-metastatic disease were further analyzed using \( t \) test and cluster analyses (unweighted pair-group method with arithmetic mean). Among 27,169 detectable genes in the samples analyzed, 728 gene expression alterations were significantly associated with metastasis and thus considered as metastasis-associated gene candidates. One of these genes, TCF12, was overexpressed in both patients diagnosed with metastasis (Fig. 1A). To ascertain whether tumor TCF12 mRNA overexpression indeed occurred in CRC, TCF12 mRNA levels were analyzed by quantitative RT-PCR from paired tumor and non-tumor tissues of 120 patients. The representative results from six patients are shown in Fig. 1B. Thirty-four (28.3%) of 120 CRC patients exhibited a reduction in TCF12 mRNA levels (<0.5-fold) in tumor tissues compared with non-tumor tissues (designated as “N > T” group). However, 33 (27.5%) of the 120 patients expressed TCF12 at a level >2-fold higher in tumor tissues than in non-tumor tissues and were designated as the patient group with “tumor TCF12 mRNA overexpression” (“N < T” group).

We also analyzed TCF12 protein levels in CRC patients. Tumor TCF12 protein overexpression was detected in 9 (23.1%) of 39 patients. The data of six representative patients are shown in supplemental Fig. 1A. In this study only 19 patients contributed sufficient specimens for preparation of both RNA and protein. Among them, 3 of 5 patients having tumor TCF12 mRNA overexpression showed elevated expression of TCF12 protein in their tumor tissues rather than adjacent non-tumor tissues; 12 of 14 patients without tumor TCF12 mRNA overexpression did not overexpress TCF12 protein in their tumor tissues either (\( p = 0.046 \), supplemental Fig. 1B). The correlation was significant in this group of patients (\( R = 0.457, p = 0.049 \), two-tailed). In addition, we also performed the immunohistochemical staining of TCF12 in the tissue sections of nine patients exhibiting tumor TCF12 protein overexpression detected by immunoblot analysis. As expected, TCF12 intensely stained in the tumor tissues rather than adjacent non-tumor parts. The representative data are shown in supplemental Fig. 1C.

**Tumor TCF12 mRNA Overexpression Is Risk Factor for CRC Metastasis**—The clinical relevance of tumor TCF12 mRNA status was investigated. As shown in Table 1, tumor TCF12 mRNA level showed no correlation with patient age, gender, tumor size, tumor site, tumor cell differentiation, or TNM staging (\( p > 0.05 \)). A further analysis revealed that tumor TCF12 mRNA overexpression was significantly correlated with tumor metastasis (\( p = 0.020 \)) but not with tumor invasion depth (\( p = 0.261 \)). Among the 33 patients with tumor TCF12 mRNA overexpression, 23 (69.7%) cases had documented metastases to lymph nodes or other visceral organs, whereas 58.0% of the patients without tumor TCF12 mRNA overexpression had tumors confined to primary sites (Table 1). Because metastasis is the major cause of CRC mortality, the patients with tumor TCF12 mRNA overexpression exhibited a poorer survival outcome (\( p = 0.014 \), Fig. 1C).

Logistic regression analyses were performed to evaluate the predictive value of tumor TCF12 mRNA overexpression for metastatic occurrence. The patient variables, including tumor
size, depth of tumor invasion, serum levels of CEA and CA19-9, and tumor TCF12 mRNA overexpression were subjected to univariate analyses (Table 2). Three variables, i.e. tumor invasion depth, tumor TCF12 mRNA overexpression, and serum CEA level, showed \( p < 0.05 \) and were, therefore, entered into a multivariate analysis. The patient group with tumor TCF12 mRNA overexpression showed a statistically significant incidence of tumor metastasis (odds ratio, 2.722, 95%; confidence

**TABLE 1**
Clinical relevance of tumor TCF12 mRNA expression status in CRC

| Patient variables | TCF12 mRNA expression status | \( p \) value* |
|-------------------|------------------------------|---------------|
|                  | \( N > T \) (\( n = 34 \)) | \( N = T \) (\( n = 53 \)) | \( N < T \) (\( n = 33 \)) |
| Age (mean ± S.D., year) | 68.9 ± 11.1 | 69.2 ± 10.0 | 68.6 ± 12.0 | 0.973 |
| Gender (male/female) | 27/7 | 40/13 | 27/6 | 0.773 |
| Tumor size (mean ± S.D., cm) | 5.3 ± 2.3 | 5.0 ± 2.2 | 4.8 ± 2.2 | 0.724 |
| Tumor site | | | | 0.588 |
| Cecum | 1 | 2 | 1 |
| Ascending colon | 1 | 5 | 5 |
| Transverse colon | 1 | 2 | 3 |
| Descending colon | 2 | 2 | 2 |
| Sigmoid | 15 | 12 | 8 |
| Rectosigmoid junction or rectum | 12 | 28 | 13 |
| Multiple sites | 2 | 2 | 1 |
| Tumor cell differentiation | | | | 0.067 |
| Well differentiation | 1 | 1 | 0 |
| Moderate differentiation | 30 | 52 | 33 |
| Poor differentiation | 3 | 0 | 0 |
| TNM stage | | | | 0.112 |
| I | 5 | 11 | 1 |
| II | 14 | 21 | 9 |
| III | 11 | 13 | 14 |
| IV | 4 | 8 | 9 |
| Tumor invasion depth | | | | 0.261 |
| Submucosa | 3 | 3 | 1 |
| Muscularis propria | 3 | 9 | 1 |
| Subserosa | 23 | 28 | 25 |
| Other organs or structures | 5 | 13 | 6 |
| Lymph node or distant metastasis | 15 | 21 | 23 | 0.020 |
| CEA (ng/ml) | 18.1 ± 54.0 | 38.7 ± 80.7 | 86.5 ± 146.5 | 0.025 |
| CA19-9 (units/ml) | 442.8 ± 204.4 | 354.3 ± 1209.5 | 233.0 ± 654.2 | 0.886 |

* Differences were considered significant if \( p < 0.05 \) (expressed as numbers in bold).
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TABLE 2
Logistic regression analyses of the predictive values of tumor TCF12 mRNA overexpression and some clinicopathologic variables for the occurrence of CRC metastasis

| Clinicopathological variables | Univariate logistic regression | Multivariate logistic regression |
|------------------------------|-------------------------------|---------------------------------|
| Tumor TCF12 overexpression (yes vs. no) | 3.257 (1.383–7.668) 0.007 | 2.722 (1.019–7.269) 0.046 |
| Tumor size (≥5 cm vs. <5 cm) | 0.814 (0.397–1.670) 0.575 | | |
| Tumor invasion depth (≥subserosa vs. <subserosa) | 7.209 (1.986–26.168) 0.003 | 4.667 (1.160–18.783) 0.030 |
| Serum CEA level (≥6 ng/ml vs. <6 ng/ml) | 2.917 (1.308–6.504) 0.009 | 1.773 (0.729–4.315) 0.207 |
| Serum CA19-9 level (≥35 units/ml vs. <35 units/ml) | 2.500 (0.912–6.851) 0.075 | | |

* CI, confidence interval. ** Differences were considered significant if \( p < 0.05 \) (expressed as numbers in bold).

A Immunoblot analysis:

B Immunoblot analysis:

C qPCR analysis:

FIGURE 2. SW480 and SW620 cells have different levels of TCF12 expression. A, immunoblot analysis was performed to detect abundant TCF12 expression in CRC cell lines HCT-8, HCT-116, SW480, SW620, and LoVo. B, a higher level of TCF12 expression was detected in SW620 cells compared with SW480 cells by immunoblot analysis. C, a higher TCF12 mRNA level was detected in SW620 cells compared with SW480 cells by qPCR analysis.

interval, 1.019–7.269, \( p = 0.046 \). In addition, the patient group with tumor invasion depth at subserosa or deeper also exhibited a significant level of tumor metastasis (\( p = 0.030 \)). The univariate correlation of serum CEA level with metastasis did not reach statistical significance after multivariate regression analysis (\( p = 0.207 \)). These analyses suggest that tumor TCF12 mRNA overexpression can be a risk factor for the development of metastasis in CRC.

TCF12 Regulates CRC Cell Migration, Invasion, and Metastasis—Besides CRC tissue specimens, we also detected TCF12 protein levels in human CRC cell lines HCT-8, HCT-116, SW480, SW620, and LoVo. Although abundant levels of TCF12 were detected in all these cell lines, we have noted that SW480 cells exhibited a least level of TCF12 (Fig. 2A). The cell line SW480 was established from the primary tumor of a CRC patient, and the cell line SW620 was established from the lymph node-metastasizing tumor from the same patient 1 year later. Reproducible data indicated that SW620 cells indeed expressed higher TCF12 protein and mRNA levels when compared with SW480 cells (Fig. 2, B and C). Furthermore, we established stable cell clones of vector transfection and shRNA-mediated TCF12-knockdown from SW620 and LoVo cells. An example data indicating shRNA-mediated TCF12 down-regulation was shown in Fig. 3A. Time-lapse photography was performed to monitor 12-h migration tracks of these cells. After analysis by Image-Pro Plus software, the data revealed that both accumulated and oriented distances of cell migration were obviously reduced in TCF12-knockdown SW620 (Fig. 3) and LoVo (Fig. 4A) cells. However, the stable cell clone of SW480 cells with ectopic TCF12 overexpression exhibited an elevated level of cell migration (Fig. 4B), suggesting that TCF12 expression was associated with the migration activity of CRC cells. Cell invasiveness was also reduced when TCF12 was knocked down in SW620 and LoVo cells (Fig. 5, A and B) but increased in TCF12-overexpressing SW480 cells (Fig. 5C). Therefore, TCF12-mediated metastatic activity was further evaluated by injecting the tail veins of mice with vector-transfected or TCF12-knockdown SW620 cells. In parallel with the data of \textit{in vitro} migration and invasion assays, the mice injected with TCF12-knockdown SW620 cells had lower levels of lung metastasis when compared with those injected with vector-transfectants (tumor nodules per mouse, 73.0 \( \pm \) 22.7 \( p = 0.001 \), Fig. 5C).

TCF12 Regulates E-cadherin Expression and Gap-junction Activity—TCF12-knockdown SW620 cells were characterized by the tight cell-cell contact, which differed from the stacked-up pattern exhibited by parental cells or vector-transfectants (Fig. 6A), suggesting that TCF12 plays a role in the regulation of cell-cell contact. E-cadherin is a marker of the epithelial phenotype and a major component of adherens junctions between cells. Because the E-cadherin gene promoter contains several E-box sites, whether TCF12 was physically associated with E-cadherin promoter or not was investigated by ChIP assay. SW620 cells, expressing a higher level of TCF12, showed a significant association of TCF12 with E-cadherin promoter, which was not detected in SW480 cells (Fig. 6B). This association was diminished when TCF12 was specifically knocked down. Furthermore, an increase in E-cadherin and an accompanying decrease in fibronectin levels were detected in TCF12-knockdown SW620 cells (Fig. 6C). A significant induction of two gap-junction components, connexin 26 and connexin 43, was also detected when TCF12 was down-regulated (Fig. 6C). Assay of gap-junction-mediated calcein transfer was performed to investigate the effect of TCF12 expression on cellular gap-junction activity. A marked increase in the number of calcein-accepting cells was found in TCF12-knockdown SW620 cells compared with vector-transfectant cells (Fig. 6D), suggesting that TCF12-knockdown restored E-cadherin-mediated cell-cell contact and favored cellular gap-
TCF12 knockdown reduces SW620 cell migration. A, the result of qPCR analysis shows a 93% reduction of TCF12 mRNA expression in a representative stable clone of TCF12-knockdown SW620 cells compared with vector-transfectant SW620 cells. B, cell migration tracks of SW620 vector-transfectant and TCF12-knockdown cells are shown. Cell migration was monitored for 12 h by time-lapse photography, and the movement tracks of 20 randomly selected vector-transfectant or TCF12-knockdown cells were analyzed by Image-Pro Plus software. C, shown is quantification of the accumulated and oriented migration distances of vector-transfectant or TCF12-knockdown cells selected in B. D, a comparison of the averages of the accumulated and oriented migration distances between vector-transfectant and TCF12-knockdown SW620 cells is shown. The data are expressed as mean ± S.D., and differences are considered significant if $p < 0.05$. The above results are representative of three independent experiments.

TCF12 Overexpression Involved in Colorectal Cancer Metastasis

TCF12 Is Co-expressed and Co-immunoprecipitable with Bmi1 and EZH2—To study the mechanism responsible for TCF12-caused E-cadherin down-regulation, we noticed a tight correlation between the levels of TCF12 and Bmi1/EZH2, i.e.
down-regulated Bmi1 and EZH2 levels were detected in TCF12-knockdown SW620 and LoVo cells, whereas elevated Bmi1 and EZH2 expression occurred in TCF12-overexpressing SW480 cells (Fig. 8A). Additionally, the nuclear extracts of SW620 and SW480 cells were immunoprecipitated with anti-TCF12 antibody, and the precipitated proteins were further subjected to immunoblot analyses with anti-Bmi1 and anti-EZH2 antibodies. The results revealed that TCF12 was co-immunoprecipitable with Bmi1 and EZH2 (Fig. 8B), suggesting that TCF12 functioned as a transcriptional repressor of E-cadherin via the complex formation with Bmi1 and EZH2.

Clinical Correlation of TCF12 Overexpression with E-cadherin Down-regulation

To clinically investigate whether TCF12 overexpression was correlated with E-cadherin down-regulation, we investigated the status of E-cadherin mRNA expression from the 120 above-studied CRC patients. As summarized in Fig. 9, the rates of tumor TCF12 mRNA overexpression and tumor E-cadherin mRNA down-regulation both increased with the staging of CRC patients. The patients with tumor TCF12 mRNA overexpression had a significant tendency to exhibit tumor E-cadherin mRNA down-regulation \( (p = 0.013) \). A further analysis indicated that the statistically significant correlation between tumor TCF12 mRNA overexpression and tumor E-cadherin mRNA down-regulation existed in the CRC patients with earlier stages (TNM1 or -2, \( p = 0.041 \)) but not late stages (TNM3 or -4, \( p = 0.251 \)). In the 59 patients with tumor metastasis, there were 15 patients exhibiting TCF12 mRNA overexpression and coupled E-cadherin mRNA down-regulation in their tumor tissues; 8 patients overexpressed TCF12 mRNA but without E-cadherin down-regulation in their tumor tissues; 18 patients had tumor E-cadherin mRNA down-regulation without tumor TCF12 mRNA overexp-
tumor metastasis. Identification of molecular risk factors for the development of CRC metastasis will not only help clarify the mechanisms mediating the progression of this disease but also provide prognostic clues for the design of more aggressive therapeutic strategies. This study used microarray analysis, qPCR, and a survey of 120 patients to identify tumor TCF12 mRNA overexpression as a risk factor for CRC metastasis. Cancer metastasis is known to be the major cause of CRC mortality. Our analysis revealed that the patient group with tumor TCF12 mRNA overexpression indeed exhibited a poorer survival outcome when compared with patients without TCF12 overexpression in tumor tissues.

TCF12 functioned in T-cell development and regulation by heterodimerizing with other class I HLH proteins (e.g. E12 and E47) (24–26). TCF12 formed complexes with class II myogenic factors (like Myf3, Myf5, and Myf6) for myogenesis (8, 27, 28). TCF12 was also up-regulated and involved in the proliferation of neural stem and progenitor cells (29). Additionally, TCF12 could be associated with the class V member Id1 to suppress E-cadherin expression during the initiation stage of renal tubular epithelial cell dedifferentiation (22). In this study, induction and down-regulation of E-cadherin expression were observed in TCF12-knockdown and TCF12-overexpressing CRC cells, respectively, suggesting that TCF12 regulated E-cadherin expression in CRC. Clinically, we observed that the rates of tumor TCF12 overexpression and tumor E-cadherin down-

DISCUSSION

CRC is one of the most common human malignancies in the world. Surgical removal of tumor is the major curative treatment for CRC patients; however, the long term survival rate is still not satisfactory because of the frequent occurrence of
regulation both increased with the staging of CRC patients. The correlation of TCF12 mRNA overexpression with E-cadherin mRNA down-regulation was also detected from our 120 CRC patients. E-cadherin is a transmembrane glycoprotein constituting the epithelial adherens junction (30), and its loss/reduction is a hallmark event required for EMT process (11). EMT is thought to be involved in cancer progression. Tumor cell migration and invasion is an initial and essential step for the development of metastasis and is controlled by a coordinated series of cellular and molecular changes that are reminiscent of EMT to enable tumor cells to dissociate and migrate from the primary tumor (31–34). Although not all molecular characteristics of EMT are observed during tumor cell migration and invasion (35), there is increasing evidence for the association of E-cadherin down-regulation with the initiation of malignant progression (11). Because our data also indicated that TCF12 regulated CRC cell migration and invasion, TCF12 overexpression could initiate CRC progression by down-regulating E-cadherin expression, eliciting EMT, and thus enhancing tumor cell migration and invasion.

A physical association of TCF12 with E-cadherin gene promoter was demonstrated by our ChIP assay. Moreover, TCF12 was co-expressed and co-immunoprecipitable with Bmi1 and EZH2, suggesting that TCF12 transcriptionally repressed E-cadherin expression via PRC1 and PRC2 participation. Several transcription factors of Snai1, ZEB, and HLH families have been reported to repress E-cadherin expression and are thought to play important roles in tumor cell progression (11). The HLH class I member TWIST1 is well characterized as an E-cadherin repressor and an EMT inducer (36). The association of TWIST1 with cancer progression has also been extensively studied (13–21). The results of our present study suggest that the HLH class I protein TCF12 can also function as a transcriptional repressor of E-cadherin and play an important role in cancer cell progression by enhancing the EMT process. However, the mechanism responsible for the increase of TCF12 mRNA expression in tumor cells is not clear. TWIST1 and ZEB1 were reportedly up-regulated upon loss of E-cadherin, and knockdown of TWIST1 expression markedly antagonized tumor cell invasiveness and the resistance to anoikis induced by loss of E-cadherin (37). It seemed that a transient loss of E-cadherin resulted in an induction of its own repressors, forming a signaling loop to maintain E-cadherin down-regulation and even induce crucial downstream effectors for the completion of metastatic cascade.

Our data also indicated that TCF12 affected the expression of connexin 26 and connexin 43, two major components of cellular gap junctions. TCF12-knockdown cells were found to express increased levels of connexin 26 and connexin 43, but cells with ectopic TCF12 overexpression indeed exhibited down-regulation of connexin 26 and connexin 43 expression. The results of calcein-transfer assay further confirmed that TCF12 indeed had an inhibitory effect on cellular gap-junction activity. It could be contributed by TCF12-induced repression of E-cadherin expression because E-cadherin constitutes cellular adherens junctions that can facilitate and maintain gap junctions (38, 39). Additionally, TCF12 could transcriptionally repress connexin 26 and connexin 43 expression via the conserved E-box sites in their promoter regions. Snai1 has been reported to be a transcriptional repressor of connexin 43 (40). Knockdown of Snai1 in embryonal carcinoma cells resulted in elevated connexin 43 expression and mesenchyme-to-epithelium transition. In MDA-MB-231 breast cancer cells, exogenous overexpression of connexin 26 or connexin 43 has been adopted to block the EMT process, resulting in an increase in cytokeratin 18 expression but a decrease in cell migration and vimentin expression (41). These results support that TCF12 functions as a regulator of EMT by affecting the expression of E-cadherin as well as that of connexin 26 and connexin 43. The change of gap-junction activity can be considered as a functional marker of TCF12-induced EMT.

Despite that the data of TCF12-knockdown and overexpression in cell culture studies have suggested that TCF12 could promote CRC metastasis via functioning as a repressor of E-cadherin expression, our clinical investigation revealed that tumor TCF12 mRNA overexpression was significantly correlated with tumor E-cadherin mRNA down-regulation in all 120 CRC patients (p = 0.013) and the patients with TNM1 or -2 (p = 0.041).

![Tumor E-cadherin mRNA Down-Regulation](image-url)

**FIGURE 9.** Tumor TCF12 mRNA overexpression is correlated with tumor E-cadherin mRNA down-regulation in the CRC patients with TNM stage 1 or 2. The status of tumor E-cadherin mRNA expression was investigated from 120 patients studied in Fig. 1. The summaries of the patient numbers with tumor TCF12 overexpression or/and tumor E-cadherin down-regulation were depicted as three panels: left, all patients; middle, TNM1 or -2 patients; right, TNM3 or -4 patients. The Pearson χ² analysis reveals that tumor TCF12 mRNA overexpression was significantly correlated with tumor E-cadherin mRNA down-regulation in all 120 CRC patients (p = 0.013) and the patients with TNM1 or -2 (p = 0.041).
mRNA overexpression had the equal ratio (18/36) to have or not E-cadherin mRNA down-regulation. Because other transcriptional factors such as TWIST1 and Snail1 can also suppress E-cadherin expression, it is probable that overexpression of TWIST1 or Snail1 could occur in our late-staging patients and account for the cases with E-cadherin mRNA down-regulation but without TCF12 mRNA overexpression. It seemed that TCF12 was involved in a complicated machinery for E-cadherin regulation.

Metastasis is a complex process by which tumor cells travel to different tissues/organs through the body’s circulation systems and is controlled by several factors that act in multiple steps. Down-regulation of E-cadherin and thus the EMT reboot can be one of the important mechanisms to enhance tumor cell migration and invasion that are often early steps for the development of metastasis. In addition to E-cadherin, other malignancy-associated genes, such as p16^INK4a and Bmi1 (14), also contain E-box elements and can, therefore, be the downstream targets of TCF12. Analysis of the gene expression profile regulated by TCF12 through transcriptional repression and activation will be a pathway to disclose additional molecules that control the occurrence of CRC metastasis.

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