TWIST1 and SNAI1 as markers of poor prognosis in human colorectal cancer are associated with the expression of ALDH1 and TGF-β1

YONG HUN KIM[1], GWANGIL KIM[2*], CHANG-IL KWON[3*], JONG WOO KIM[3], PIL WON PARK[3] and KI-BAIK HAHM[3]

[1]Department of Medicine, The Graduate School, CHA University; [2]Department of Pathology, and [3]Digestive Disease Center, Bundang CHA Medical Center, CHA University, Bundang-gu, Seongnam 463-712, Republic of Korea

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Abstract. Epithelial-mesenchymal transition (EMT) is an important factor in cancer invasiveness and metastatic progression. During EMT, cancer cells acquire stem cell properties. The role of EMT and stemness in colon cancer has not been fully understood. We aimed to demonstrate the clinical significance of EMT and the stem cell phenotype in colorectal cancer. Two hundred and thirty-one surgically resected colon cancer cases were included in the present study. mRNAs of E-cadherin, TWIST1 and SNAI1 were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) (n=109). Immunohistochemical staining was performed for six markers (ALDH1, TGF-β1, E-cadherin, β-catenin, TWSIT1 and SNAI1) (n=231). We assessed clinicopathological characteristics according to the expression of the stem cell phenotype and EMT markers. Based on the results of qRT-PCR, TWIST1 and SNAI1 significantly influenced node metastasis (P=0.04 and P=0.02, respectively). High TWIST1 and SNAI1 mRNA expression was associated with poor overall survival according to the univariate analysis (P<0.01 and P=0.01, respectively) and the multivariate analysis (P=0.04 and P=0.04, respectively). ALDH1 expression as detected by immunohistochemical staining was associated with high nodal stage, advanced clinical stage, lymphatic invasion and poor survival (P=0.01, P=0.04, P<0.05 and P<0.01, respectively) and with the expression of TGF-β1 and β-catenin. In conclusion, in human colorectal cancer, the EMT markers TWIST1 and SNAI1 are suggested as important markers of poor prognosis. Their expression is associated with the expression of putative stem cell marker ALDH1, and ALDH1 is associated with the expression of TGF-β1.

Correspondence to: Professor Gwangil Kim, Department of Pathology, Bundang CHA Medical Center, CHA University, 59 Yatap-ro, Bundang-gu, Seongnam 463-712, Republic of Korea
E-mail: blacknw@cha.ac.kr

Professor Chang-Il Kwon, Digestive Disease Center, Bundang CHA Medical Center, CHA University, 59 Yatap-ro, Bundang-gu, Seongnam 463-712, Republic of Korea
E-mail: endoscopy@cha.ac.kr

*Contributed equally

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Introduction

Colorectal cancer, one of the most common epithelial carcinomas originating from epithelial cells, is the major cause of cancer-related mortality in industrialized countries (1). Despite the development of numerous surgical techniques and new treatment methods, metastatic colorectal cancer is associated with a low (~10%) 5-year survival rate and accounts for 90% of colon cancer-related deaths (2,3). Primary tumors are known to metastasize through the process of epithelial-mesenchymal transition (EMT) (4), which is an important step by which epithelial cells transform into mesenchymal cells during embryonic development, but loss of cell polarity, decreased cell to cell adhesion, increased migratory ability and increased invasive property are caused by the aberrant activation of EMT in cancer cells (5-7). It is essential, therefore, to understand the process of EMT that occurs during the development of colon cancer, in addition to understanding the mechanisms involved in the progression of carcinoma, to be able to establish objectives for the prevention and treatment of metastasis. The process of EMT is accompanied by various changes, such as repression of epithelial markers, abnormal translocation of β-catenin, and upregulation of mesenchymal markers, as well as expression of and interaction between related proteins or so-called EMT regulators, such as TWIST1 and SNAI1 (SNAIL), but there is still a lack of understanding in individual carcinomas (6,8).

According to previous research, cancer cells appear to acquire a stem cell-like phenotype during the process of EMT (9). Cancer stem cells have features of self-renewal and tumor-initiating capacity, and the resultant tumors can

be defined as cells having high heterogeneity of a primary tumor. It has been suggested that, unlike stem cells in the crypt of normal colonic mucosa, the microenvironment of cancer stem cells hosts variants of diverse self-renewable signaling pathways, such as the Wnt/β-catenin, TGF-β, Notch and Hedgehog signaling pathways (10). Cancer stemness is a concept introduced to describe cancer cell resistance to conventional chemoradiation therapies and has been suggested to have a close relationship with EMT in breast and prostate cancer (11-13). ALDH1 has been found to be a valuable marker in previous studies for identifying breast and colon cancer stem cells (13-16). It has been speculated that the extent of the expression of cancer stem cell phenotype markers may be associated with the clinical prognosis of colon cancer, but without sufficient clinical research to support it.

In the present study, we aimed to determine whether EMT markers and the extent of expression of stem cell phenotype markers are associated with the histological features and clinical prognosis of colon cancer patients.

Materials and methods

Patients and tissue samples. After obtaining approval from the Institutional Review Board of Bundang CHA Medical Center, 231 patients with primary colon cancer recruited between March 2002 and October 2006 were included in the present study. The patients underwent surgery for colon cancer at our center, and the collected samples were examined and classified by GI pathologists at the center. The 231 patients consisted of 130 males and 101 females, ranging in age from 31 to 95 years (mean age, 61.8). The patients were followed up for a median of 71 (1-106) months. Of the 231 cases, 23 cases were mucinous adenocarcinoma and 37 cases were right colon cancer. Other clinical parameters such as gender, clinical stage and survival time (months) are detailed in Tables I and II. Tissue microarray was used for the 231 cases of paraffin-embedded tissue blocks. For 109 cases with available normal tumor-paired samples, fresh snap-frozen samples were used for quantitative analysis of mRNA expression. In order to select cases containing at least 90% tumor cells, frozen tissues were subjected to histological evaluation with H&E staining. Clinical stages were determined according to the AJCC staging system, 7th edition by a clinician who reviewed the surgical pathology reports and clinical information (17).

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR). RNA was extracted from 109 fresh snap-frozen samples of colon cancer tissues and from the paired normal tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from the extracted RNA (1 μg) using the SuperScript III kit (Invitrogen). Each cDNA sample was run three times independently using the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Gene-specific primers and probes for the TaqMan gene expression assay were purchased from Applied Biosystems (Paisley, UK). This included three genes: E-cadherin (Hs00170423_m1), TWIST1 (Hs00361186_m1) and SNAI1 (Hs00195591_m1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) was used for normalization. The PCR reaction mix consisted of a volume of 20 μl and was composed of 10 μl 2X TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μl of primers and probe kit, 1 μl of cDNA and 8 μl of diethyl pyrocarbonate water. The reverse transcription was carried out for 15 sec at 90˚C, repeated 40 times for 1 min at 60˚C, and then was treated for 2 min at 50˚C and for 10 min at 95˚C. Initial copy number at real-time PCR analysis was quantified based on the threshold cycle (Ct), and target genes were normalized against GAPDH. Relative quantification of mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemical analysis. For the tissue microarray of the 231 cases, tissue cores were obtained at a 2-mm diameter from expression regions on formalin-fixed paraffin-embedded blocks using a manual tissue microarray kit (Unitma, Quick-Ray; Unitech Science, Seoul, Korea). The obtained tissues consisted of three cancer tissue cores from different foci and one matched normal tissue core. Immunohistochemical staining was performed on 5-μm sections cut from formalin-fixed, paraffin-embedded tissue using mouse monoclonal anti-ALDH1 (1:50) (BD Biosciences, La Jolla, CA, USA), rabbit polyclonal anti-TGF-β1 (1:100) (Novus Biologicals, Littleton, CO, USA), mouse monoclonal anti-E-cadherin (1:100), mouse monoclonal anti-β-catenin (1:100) (both from BD Biosciences), rabbit polyclonal anti-TWIST1 (1:200) and rabbit polyclonal anti-SNAI1 (1:100) (both from Novus Biologicals). The visualization system used was the BenchMark XT with heat-induced epitope retrieval (CC1 solution) (both from Ventana). Sections were incubated with primary antibodies for 32 min at 37˚C. Staining was detected with the ultraView Universal DAB detection kit (Ventana). Immunohistochemical content was scored depending on the extent and intensity of staining, as previously described (18). In brief, the intensity of staining was graded according to a 4-tiered scale of 0 to 3 (with 3 as the most intense staining). The extent of positive immunoreactivity was graded according to the percentage of stained cells in the region of interest: 0 points for 0%; 1 point for <20%; 2 points for 20-50% and 3 points for >50%. An overall score was obtained from the sum of the intensity and the extent of the positive-staining. Cases with a final score of >3 were defined as positive. Cases with cytoplasmic and/or nuclear localization of β-catenin were considered abnormal. All staining was separately scored in a blinded manner by two trained researchers (Y.K. and C.K.) and one pathologist (G.K.). In the case of disagreement in interpretation, the results were discussed by all three researchers and a consensus was reached for each case.

Statistical analysis. Quantitative values of qRT-PCR were presented as means ± standard deviation (SD) or median. The Mann-Whitney U test was used for quantitative comparison of mRNAs with respect to various clinical and histological parameters. The Chi-square test was used to compare immunohistochemical staining with clinical and pathological indices. For correlation of EMT and stemness markers, Spearman's rank correlation was used. Patient overall survival was determined from official death records by the Korea Statistics Promotion Institute. Survival curves were calculated using the Kaplan-Meier method, and differences between the survival curves were analyzed using log-rank test. Multivariate analysis

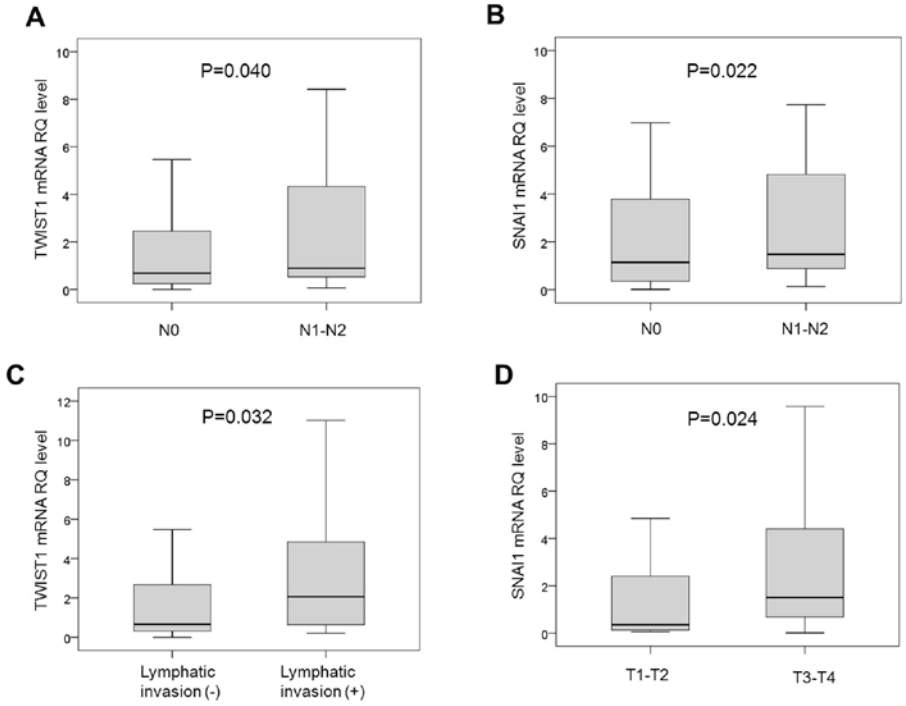

Figure 1. Correlation between relative mRNA quantification (RQ) of TWIST1 and/or SNAI1 and (A and B) nodal stage, (C) lymphatic invasion and (D) tumor stage. RQ is expressed as fold-change using the $2^{-\Delta\Delta Ct}$ method in real-time PCR analysis.

of independent prognostic factors of survival was performed using Cox's proportional hazard regression model. All statistical analyses were performed with IBM® SPSS® Statistics (version 19.0.0), and P-value <0.05 was considered to indicate a statistically significant result.

Results

Correlation between the relative quantification (RQ) of mRNA expression of EMT markers and clinicopathologic characteristics of the colorectal cancer cases. The mean values of RQ for E-cadherin, TWIST1 and SNAI1 were 72.73±293.62 (median, 3.27), 8.13±31.7 (median, 0.73) and 3.47±6.06 (median, 1.48), respectively in the 109 cases. Table I summarizes the differences in the mean values of RQ of each marker according to the clinicopathological characteristics. The mean values of RQ of E-cadherin, TWIST1 and SNAI1 were not significantly different in regards to gender, age, location of the colon cancer, number of primary lesions and pathological diagnosis. When samples excluding mucinous adenocarcinoma were divided into a well to moderate differentiation group and a poor to undifferentiated group, the two subgroups were not significantly different in regards to the mean values of RQ for each marker. The mean values of RQ for TWIST1 and SNAI1 were significantly higher in the subgroup without nodal invasion (N0) when compared to that with nodal invasion (N1-3) (P=0.040 and P=0.022, respectively) (Fig. 1A and B, respectively). The mean value of RQ for TWIST1, but not for those of E-cadherin and SNAI1, was significantly high in the presence of lymphatic invasion (P=0.032) (Fig. 1C). SNAI1 showed a higher mean value of RQ in T3-4 stage than in T1-2 stage cases (P=0.024) (Fig. 1D), and the mean values of RQ for E-cadherin, TWIST1 and SNAI1 were not significantly different depending on whether vascular invasion or neural invasion was present or not.

Immunohistochemical staining for EMT and stem cell markers. qRT-PCR was performed for mRNAs of EMT and stem cell markers, and immunohistochemical staining was conducted to validate the possibility of EMT marker expression in the surrounding mesenchymal cells of the tumor cells. Representative images of the immunohistochemical staining for each marker are presented in Fig. 2. ALDH1 was positive in ~5% of cells at the crypt of the normal colon epithelium. E-cadherin and β-catenin were strongly expressed along the cell membranes of normal epithelium. TGF-β1, TWIST1 and SNAI1 were not expressed in normal epithelial cells. The relationship between immunohistochemical staining and the clinicopathological characteristics of our 231 cases is summarized in Table II. ALDH1, TGF-β1, E-cadherin, nuclear β-catenin, SNAI1 and TWSIT1 were not significantly different in terms of immunohistochemical staining according to gender, age, location of the lesion and number of primary lesions. The extent of nuclear β-catenin expression was significantly higher in the mucinous adenocarcinoma than in the adenocarcinoma cases (P=0.046). When carcinomas excluding mucinous adenocarcinoma were divided into a well to moderately differentiated group and a poor to undifferentiated group, the former was observed to express nuclear β-catenin and TWIST1 significantly at a higher extent (P=0.042 and P=0.022, respectively). Nuclear β-catenin expression was higher in T1-2 stage than in T3-4 stage cases (p=0.033), and ALDH1 and TGF-β1 were expressed at a higher extent in N1-3 stage than in N0 stage cases (P=0.010 and P=0.032, respectively). In terms of lymphatic invasion, vascular invasion and neural invasion analysis, ALDH1 was highly expressed in

Table I. Clinicopathological features and the mean values of the relative quantification of mRNA expression of EMT markers in the colorectal cancer cases.

	n	E-cadherin (mean ±SD)	TWIST1 (mean ±SD)	SNAI1 (mean ±SD)
Total no. of samples	109	72.73±293.62	8.13±31.7	3.47±6.06
Gender				
Male	64	2.97±298.91	1.28±34.81	1.53±3.84
Female	45	3.64±281.93	0.66±26.26	1.48±8.17
P-value		0.751	0.475	0.929
Age (years)				
≤50	20	105.63±363.01	3.53±6.22	3.67±4.02
>50	89	65.34±273.32	9.17±34.72	3.42±6.4
P-value		0.362	0.670	0.568
Location of colon cancer				
Right colon	23	3.64±58.24	0.66±23.9	1.59±2.72
Left colon	86	3.22±326.53	0.78±33.31	1.48±6.4
P-value		0.832	0.469	0.741
Primary lesion				
Single	105	3.37±297.47	0.73±29.95	1.48±5.95
Multiple	4	1.46±4.19	1.12±54.68	4.47±7.17
P-value		0.203	0.573	0.375
Pathological diagnosis				
Adenocarcinoma	93	3.16±312.53	0.8±31.73	1.45±6.21
Mucinous adenocarcinoma	16	5.91±118.63	0.53±30.58	1.99±4.81
P-value		0.132	0.137	0.177
Differentiation[b]				
Well to moderate	86	3.23±324.34	0.81±32.45	1.38±3.52
Poor to undifferentiated	7	1.69±8.22	0.53±20.71	2.38±17.3
P-value		0.531	0.432	0.541
Tumor stage				
T1-2	11	1.81±7.87	0.54±2.79	0.35±1.58
T3-4	98	3.62±307.3	0.78±33.19	1.48±6.4
P-value		0.069	0.073	0.024[a]
Nodal stage				
N0	56	3.44±332.95	0.68±15.64	0.78±2.89
N1-3	53	3.27±241.82	0.89±41.84	1.6±7.94
P-value		0.712	0.040[a]	0.022[a]
Clinical stage				
Stage I-II	54	3.23±258.89	0.68±15.92	1.14±2.92
Stage III-IV	55	3.37±320.05	0.89±42.12	1.59±7.84
P-value		0.341	0.057	0.064
Lymphatic invasion				
Negative	84	3.1±232.07	0.66±35.28	1.5±3.8
Positive	25	1±0.48	2.06±12.53	1.48±10.35
P-value		0.104	0.032[a]	0.668
Vascular invasion				
Negative	96	3.23±310.59	0.71±33.53	1.48±6.22
Positive	13	16.52±23.15	0.89±3.24	1.6±4.34
P-value		0.736	0.870	0.483
Neural invasion				
Negative	102	3.28±301.61	0.69±32.18	1.51±3.94
Positive	7	7.62±7.1	0.88±17.71	3.52±20.14
P-value		0.415	0.061	0.781

Data were analyzed using the Mann-Whitney U test. [a]P<0.05. [b]Except mucinous adenocarcinoma. EMT, epithelial-mesenchymal transition.

Table II. Relationship between the clinicopathological features and positive immunohistochemical expression of EMT and stemness markers (ALDH1, TGF-β1, E-cadherin, β-catenin, TWIST and SNAI1) in the 231 colorectal cancer patients.

	n	ALDH1 n (%)	TGF-β1 n (%)	E-cadherin n (%)	Nuclear β-catenin n (%)	TWIST1 n (%)	SNAI1 n (%)
Gender							
Male	130	26 (20.0)	40 (30.8)	88 (67.7)	66 (50.8)	37 (28.5)	33 (25.4)
Female	101	16 (15.8)	24 (23.8)	68 (67.3)	50 (49.5)	28 (27.7)	22 (21.8)
P-value		0.416	0.238	0.953	0.849	0.901	0.524
Age (years)							
≤50	41	4 (9.8)	9 (22.0)	26 (63.4)	15 (36.6)	8 (19.5)	11 (26.8)
>50	190	38 (20.0)	55 (28.9)	130 (68.4)	101 (53.2)	57 (30.0)	44 (23.2)
P-value		0.179	0.364	0.535	0.054	0.176	0.617
Location of colon cancer							
Right colon	37	8 (21.6)	6 (16.2)	27 (73.0)	15 (40.5)	10 (27.0)	9 (24.3)
Left colon	194	34 (17.5)	58 (29.9)	129 (66.5)	101 (52.1)	55 (28.4)	46 (23.7)
P-value		0.554	0.088	0.441	0.199	0.870	0.936
Primary lesion							
Single	222	39 (17.6)	59 (26.6)	149 (67.1)	112 (50.5)	61 (27.5)	52 (23.4)
Multiple	9	3 (33.3)	5 (55.6)	7 (77.8)	4 (44.4)	4 (44.4)	3 (33.4)
P-value		0.212	0.120	0.503	0.724	0.267	0.448
Pathological diagnosis							
Adenocarcinoma	208	35 (16.8)	57 (27.4)	143 (68.8)	109 (52.4)	59 (28.4)	50 (24.0)
Mucinous adenocarcinoma	23	7 (30.4)	7 (30.4)	13 (56.5)	23 (44.4)	6 (26.1)	6 (21.7)
P-value		0.108	0.760	0.235	0.046[a]	0.818	0.806
Differentiation[b]							
Well-moderate	195	34 (17.4)	55 (28.2)	137 (70.3)	106 (54.4)	59 (30.3)	46 (23.6)
Poor-undifferentiated	13	1 (7.7)	2 (15.4)	6 (46.2)	3 (30.4)	0 (0.0)	4 (30.8)
P-value		0.700	0.521	0.064	0.042[a]	0.022[a]	0.517
Tumor stage							
T1-2	24	4 (16.7)	7 (29.2)	15 (62.5)	17 (70.8)	7 (29.2)	5 (20.8)
T3-4	207	38 (18.4)	57 (27.5)	141 (68.1)	99 (47.8)	58 (28.0)	50 (24.2)
P-value		0.839	0.866	0.578	0.033[a]	0.906	0.718
Nodal stage							
N0	113	13 (11.5)	24 (21.2)	77 (68.1)	62 (54.9)	28 (24.8)	26 (23.0)
N1-3	118	29 (24.6)	40 (33.9)	79 (79.0)	54 (45.8)	37 (31.4)	29 (24.6)
P-value		0.010[a]	0.032[a]	0.847	0.167	0.266	0.780
Clinical stage							
Stage I-II	111	14 (12.6)	25 (22.5)	76 (68.5)	61 (55.0)	29 (26.1)	26 (23.4)
Stage III-IV	120	28 (23.3)	39 (32.5)	80 (66.7)	55 (45.8)	36 (30.0)	29 (24.2)
P-value		0.035[a]	0.090	0.81	0.166	0.513	0.895
Lymphatic invasion							
Negative	176	27 (15.3)	48 (27.3)	122 (69.3)	94 (53.4)	49 (27.8)	41 (23.3)
Positive	55	15 (27.3)	16 (29.1)	34 (61.8)	22 (40.4)	16 (29.1)	14 (25.5)
P-value		0.045[a]	0.793	0.300	0.083	0.857	0.743
Vascular invasion							
Negative	182	30 (16.5)	51 (28.0)	127 (69.8)	89 (48.9)	48 (26.4)	37 (20.3)
Positive	49	12 (24.5)	13 (26.5)	29 (59.2)	27 (55.1)	17 (34.7)	18 (36.7)
P-value		0.197	0.836	0.160	0.441	0.250	0.017[a]
Neural invasion							
Negative	209	37 (17.7)	55 (26.3)	144 (68.9)	106 (50.7)	58 (27.8)	52 (24.9)
Positive	22	5 (22.7)	9 (40.9)	12 (54.5)	10 (45.5)	7 (31.8)	3 (13.6)
P-value		0.564	0.146	0.171	0.639	0.687	0.301
Total	231	42	64	156	116	65	55

Data were analyzed using χ^2 test or Fisher's exact test. [a]P<0.05. [b]Except mucinous adenocarcinoma. EMT, epithelial-mesenchymal transition.

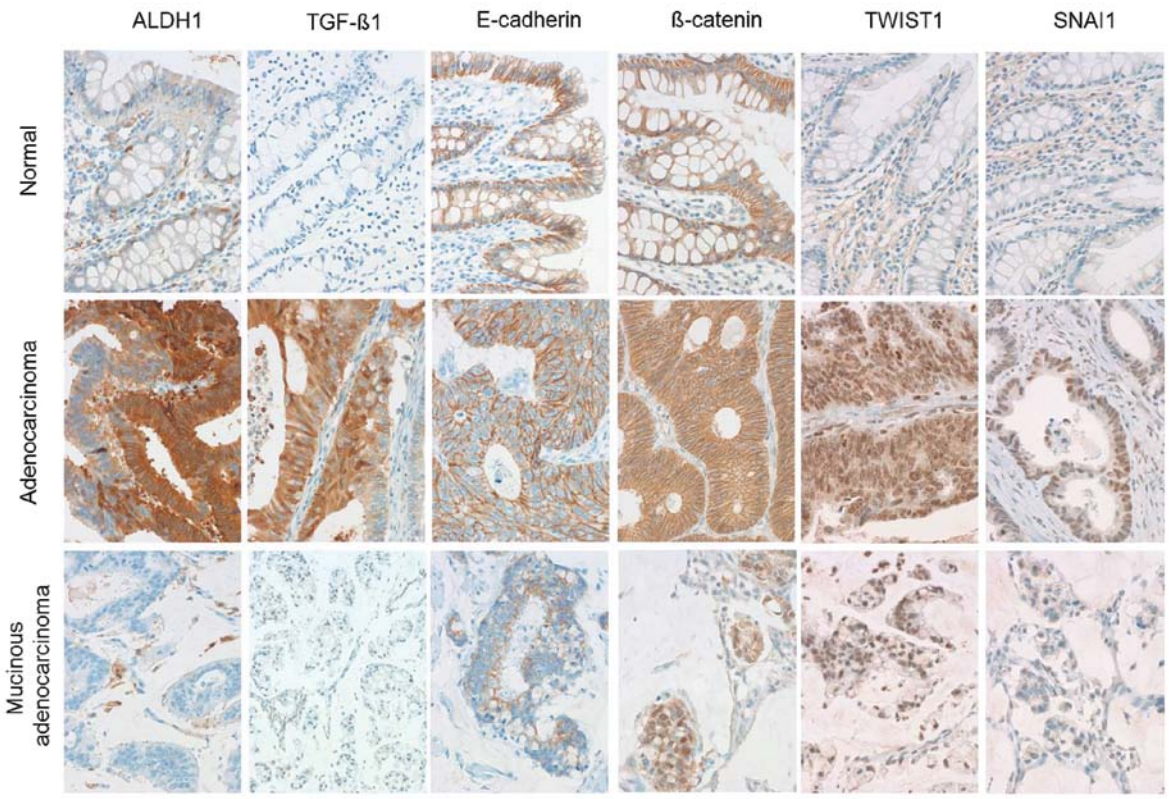

Figure 2. Immunohistochemical staining for ALDH1, TGF-β1, E-cadherin, β-catenin, TWIST1 and SNAI1 in human colon cancers. E-cadherin and β-catenin were strongly expressed along the cell membranes of normal epithelium. The extent of nuclear β-catenin expression was significantly higher in the mucinous adenocarcinomas than that in the adenocarcinomas.

the presence of lymphatic invasion (P=0.045) and SNAI1 in the presence of vascular invasion (P=0.017).

Correlation analysis of EMT and stem cell markers. Spearman's rank correlation was used to test the correlation between EMT and stemness markers (Table III). The mRNA expression levels of E-cadherin and TWIST1 were negatively correlated, while E-cadherin mRNA expression and immunohistochemical staining were positively correlated (P=0.045 and P=0.012, respectively). The mRNA expression of TWIST1 was positively correlated with the extent of expression of ALDH1, TGF-β1 and TWIST1 according to immunohistochemical staining (P=0.001, P=0.010 and P=0.004, respectively). Immunohistochemical staining of ALDH1 was significantly associated with that of TGF-β1 and nuclear β-catenin (P=0.018 and P=0.010, respectively), and immunochemical staining of TGF-β1 was positively correlated with that of TWIST1 (P=0.017).

Correlation between patient survival and EMT and stemness markers. EMT and stemness markers and overall survival were tested using univariate and multivariate analyses. Kaplan-Meier univariate analysis was performed according to the median values of RQ as cut-offs (i.e. high vs. low RQ) for E-cadherin, TWIST1 and SNAI1 mRNAs and overall survival. Cases that had high RQ for TWIST1 and SNAI1 mRNAs showed significantly lower overall survival. The mean survival of the group with high RQ of TWIST1 mRNAs was 45.1 months, which was significantly different from the mean survival of the group with a low mean RQ of TWIST1 mRNAs (53.5 months, P=0.014, log-rank test) (Fig. 3A); the mean survival of the group with a high mean RQ of SNAI1 mRNAs was 45.1 months, which was significantly different from 53.5 months in the lower group (P=0.002, log-rank test) (Fig. 3B). In the multivariate analysis, TWIST1 and SNAI1 were found to be independent significant prognostic factors for poor overall survival (P=0.041 and P=0.039, respectively; Cox's proportional hazards regression model) (Table IV). In the Kaplan-Meier univariate analysis of overall survival and immunohistochemical staining of ALDH1, TGF-β1, E-cadherin, β-catenin, TWIST1 and SNAI1, overall survival was significantly different only for ALDH1 expression (P=0.003, log-rank test), but the significance was lost in the multivariate analysis (P=0.232, Cox's proportional hazards regression model).

Discussion

EMT and cancer stem cells are important pathophysiological concepts for tumorigenesis and metastasis of cancer. Mani *et al* confirmed the role of TWIST1 and SNAI1 as EMT regulators by demonstrating EMT induction and stem cell properties when TWIST1 and SNAI1 were overexpressed in immortalized human mammary epithelial cells (9). TWIST1 and SNAI1 are known to be controlled independently and to promote EMT through collaboration as well (19,20). In previous studies, TWIST1 overexpression was observed not only in breast, prostate, lung, uterine and skin cancers but also in upper gastrointestinal tract cancers of the esophagus

Table III. Correlation between mRNA expression, immunohistochemical staining of EMT and stemness markers in the colorectal cancer cases.

	mRNA expression			Immunochemical staining					
Rho value	E-cadherin	TWIST1	SNAI1	ALDH1	TGF-β1	E-cadherin	β-catenin	TWIST1	SNAI1
E-cadherin	1	-1.93	0.05	0.01	-0.13	0.24	0.01	0.05	0.16
P-value		0.045[a]	0.636	0.960	0.181	0.012[a]	0.928	0.636	0.101
TWIST1		1	0.14	0.31	0.25	0.03	0.14	0.27	-0.01
P-value			0.153	0.001[a]	0.010[a]	0.788	0.152	0.004[a]	0.940
SNAI1			1	0.18	0.07	-0.06	0.12	-0.7	0.16
P-value				0.058	0.504	0.509	0.218	0.450	0.101
ALDH1				1	0.23	0.05	0.25	0.07	0.13
P-value					0.018[a]	0.628	0.010[a]	0.455	0.195
TGF-β1					1	-0.04	0.09	0.23	-0.010
P-value						0.655	0.346	0.017[a]	0.916
E-cadherin						1	0.14	0.03	0.11
P-value							0.162	0.739	0.260
β-catenin							1	-0.89	0.15
P-value								0.363	0.121
TWIST1								1	-0.09
P-value									0.377

Data were analyzed using the Spearman's rank statistical test. [a]P<0.05. EMT, epithelial-mesenchymal transition.

Table IV. Correlation of patient survival and EMT and stem cell markers in the colorectal cancer cases.

Variables	Univariate analysis[b]	Multivariate analysis[c]	Hazard ratio	95% CI
E-cadherin	0.859	0.897	0.95	0.46-1.97
TWIST1	0.002[a]	0.039[a]	2.29	1.04-5.00
SNAI1	0.014[a]	0.041[a]	2.11	1.03-4.33
Differentiation	0.023[a]	0.193	2.12	0.68-6.59
Stage (I, II vs. III+IV)	<0.0001[a]	0.001[a]	3.65	1.66-8.00

CI, confidence interval. [a]P<0.05. [b]Log-rank test. [c]Cox's proportional hazards model. EMT, epithelial-mesenchymal transition.

and stomach, with significant outcomes in terms of invasiveness (21-24). However, the role of TWIST1 overexpression is not yet clear in colorectal types of cancers, and there has been only a limited number of studies investigating the association between the degree of TWIST1 expression and survival in human colon cancer cases. Valdés-Mora *et al* suggested that TWIST1 overexpression in colon cancer is associated with nodal invasion, but unlike our study they did not confirm the association with overall survival (22). In addition, they concluded that TWIST1 expression was significantly higher in males and did not lead to a difference in lymphovascular invasion. They only determined the mRNA level of TWIST1 by using real-time PCR without validation of protein expression. In addition, they used a relatively small sample size of 54 colon cancer patients which included twice as many men as women. We found that higher expression of TWIST1 mRNA was associated with lymph node metastasis and poor overall survival in the 109 patients with colorectal carcinomas and their mRNA expression was validated by immunohistochemical staining in an expanded population of 231 colorectal carcinoma cases.

The Snail family, a group of zinc-finger transcription repressors, is known to play an important role in EMT by inhibiting the same cell junction component as E-cadherin (25). Shioiri *et al* suggested that the expression of SLUG (SNAI2), one of the Snail family members, was an independent prognostic factor of colon cancer (26). SNAI1, with higher affinity to E-cadherin and other target genes than SNAI2, is a candidate prognostic factor of colon cancer and is known to be significantly associated with colon cancer invasiveness and metastasis (27,28). Although the association between SNAI1 and E-cadherin was not found in the present study, we found that higher expression of SNAI1 mRNA was associated with lymph node metastasis and higher T stages. Moreover, we confirmed that overexpression of SNAI1 mRNA is a poor prognostic factor in terms of patient overall survival.

Taken together, our results support the hypothesis that TWIST1 and SNAI1 overexpression in colon cancer stimulates the process of EMT, and the resultant lymphatic and nodal invasion affects the progression of the tumors and patient survival. Since previous studies suggest that EMT is induced by cancer stem cells (9), we analyzed the association of various EMT markers and putative colorectal cancer stem

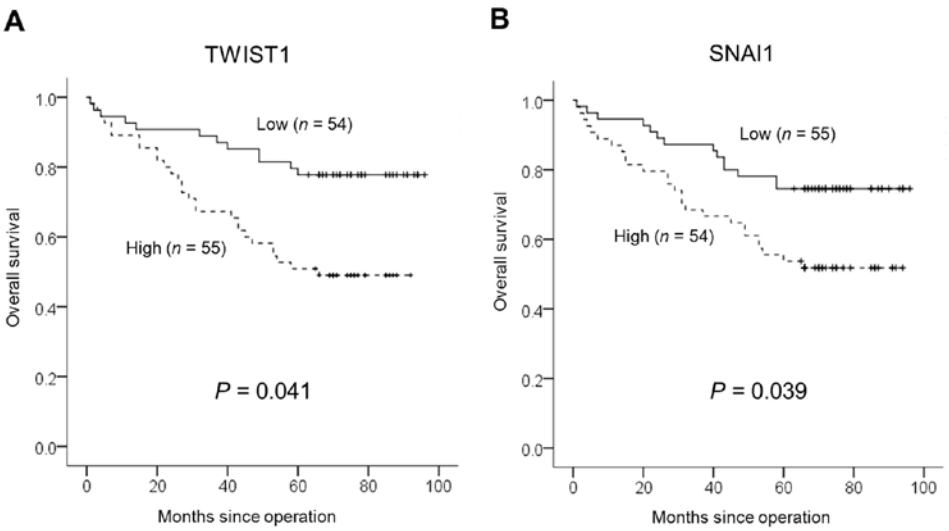

Figure 3. Kaplan-Meier overall survival curves of the human colon cancer cases according to TWIST1 and SNAI expression. High relative quantification (RQ) of mRNAs of TWIST1 and SNA1 indicates poorer overall survival.

cell markers. The cancer stem cell hypothesis, first introduced by Rudolf Virchow, was confirmed by Al-Hajj *et al* (14) in breast cancer, which is an epithelium-derived tumor, and then by O'Brien *et al* (29) who confirmed the presence of colon cancer stem cells by transplanting $CD133^+$ human colon cancer-initiating cells to immunodeficient mice. However, later reports of the non-specificity of CD133 expression in stem cells motivated us to focus on ALDH1 (30). Highly active ALDH1 increases the resistance to oxidative insults, thereby increasing the resistance to chemotherapies, and is suspected to play an important role in the recurrence and survival of carcinomas. In the present study, higher expression of ALDH1 immunostaining in colorectal cancer tissues was significantly associated with higher clinical and nodal stages, lymphatic invasion and worse overall survival. However, the association between ALDH1 expression and overall survival was significant only in the univariate analysis (P=0.003). One of the possible reason is that the expression of ALDH1 was highly correlated with and dependent on the expression of TWIST1 as shown in Table III. Our results showed that TWIST1 expression had a better association with expression of other EMT and stem cell markers than SNAI1. This may be because SNAI1 is less stable inside the cell nucleus when compared with TWIST1, although the exact half-life is unknown (31). Furthermore, in previous *in vitro* studies, TWIST1 was found to induce SNAI1 and other EMT regulators; Weinberg *et al* even suggested TWIST1 as the master transcription factor related to EMT (32,33).

In order to explain the association between EMT and cancer stem cells, we examined known EMT-related cytokines and the stem cell pathway. TGF-β is known as a cytokine that plays an important role in transcriptional activation of the Snail family, TWIST and others as well as in the regulation of cancer stem cells (34). We observed a positive correlation between TGF-β1 and ALDH1 in the immunohistochemical staining. Yet, TGF-β1 expression was not associated with the prognosis of the colorectal cancer. Increased nuclear β-catenin, the key effector of the Wnt pathway, affects EMT, cell to cell adhesion and stem cell phenotype (35). In the present study, nuclear β-catenin was expressed to a significantly higher extent in well to moderately differentiated carcinomas. Although nuclear β-catenin was identified to a greater extent in tumors with lower T-stage and higher ALDH1 expression, it was not associated with patient survival. Accordingly, our results suggest that TWIST1 is activated in human colon cancer by TGF-β, which is known to induce pro-EMT signaling stimulus and a stemness phenotype, and that activated TWIST1 regulates the initiation of EMT by suppressing E-cadherin, activates the EMT process by inducing SNAI1, and causes a stemness phenotype by inducing the expression of ALDH1. In addition, we propose that ALDH1 induces colon cancer proliferation by means of increasing nuclear translocation of β-catenin.

The present study had some limitation as functional experiments revealing causal relationship among factors were not performed. One more limitation was that the identification of EMT and stem cell markers in metastatic lesions was not shown. Nevertheless, the possibility of controlling TWIST1 and SNAI1 is an appropriate strategy in the treatment of colorectal cancers. In prostate cancer, downregulation of TWIST1 activates apoptosis and promotes chemosensitization to Taxol by downregulating the BCl-2/Bax ratio, while overexpression of TWIST1 inhibits paclitaxel-induced apoptosis in nasopharyngeal cancer (36,37). SNAI1 overexpression is also known to induce chemoresistance to oxaliplatin in colon cancer (38). TGF-β-mediated control of EMT markers and the stemness phenotype affect cancer metastasis and invasiveness. Clinical studies on ligand trap, antisense oligonucleotide and small molecule receptor kinase inhibitors, which inhibit TGF-β, are currently underway, and further studies are expected to determine the role of such agents in the inhibition of TWIST1 and SNAI1 expression and inhibition of EMT (39).

In conclusion, we demonstrated that overexpression of TWIST1 and SNAI1, both as EMT markers, is associated with poor overall survival in patients with colorectal cancers. Moreover, their expression was correlated with the expression of TGF-β1 and the cancer stem cell phenotype. Our results

suggest that controlling the EMT process through TWIST1, SNAI1 or TGF-β1 in colorectal cancers can be a possible therapeutic target of cancer stem cells. Further studies concerning the regulation of the process of EMT with functional experiments are warranted.

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