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Extensive alteration in the expression profiles of TGFB pathway signaling components and TP53 is observed along the gastric dysplasia-carcinoma sequence

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Summary. Aims: The expression patterns of TGFB signaling proteins, such as TGFB1/2, TGFBR1(ALK5), TGFBR2, SMAD1/2/3, SMAD2/3, SMAD4, SMAD7, and of downstream targets of TGFB signaling, CDKN1A ($p21^{CIP1}$), CDKN1B ($p27^{KIP1}$), MYC, CDC25A, TP53, and RELA (p65NF-kB) were investigated in gastric carcinomas and other gastric lesions. Methods and results: A total of 112 gastric carcinomas, 37 dysplasias, 54 intestinal metaplasias, 29 chronic atrophic gastritis and 54 normal gastric epithelium were analyzed by tissue microarray-based immunohistochemical analysis. Extensive changes in expression profiles of these proteins were observed. Three types of expression patterns were observed along the normal epithelium-atrophic gastritis-dysplasia-carcinoma sequence. (1) Expression of TGFB1/2, TGFBR1, MYC, and TP53 continually increased along this sequence. (2) Expression of SMAD4, CDKN1A, SMAD1/2/3, SMAD2/3, and CDKN1B was enhanced in dysplasia but decreased in carcinoma. (3) Expression of TGFBR2, SMAD7, RELA, and CDC25A was enhanced in dysplasia and the enhanced level was maintained in carcinoma. In addition, we also evaluated the clinical significance of the expression of TGFB signaling proteins in gastric carcinoma. TGFB and MYC were positively correlated with advanced stages, whereas SMAD1/2/3 and SMAD4 were strongly associated with earlier stages. Conclusions: The extensive change in expression of TGFB signaling components is implicated during tumorigenesis of gastric neoplasias.

Key words: Gastric carcinoma, Gastric dysplasia, Intestinal metaplasia, TGFB, Immunohistochemistry

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Introduction

The sequence of changes leading to gastric carcinoma (GC) is chronic atrophic gastritis-dysplasia (intra-epithelial neoplasia)-carcinoma (Ho, 1996; Genta and Rugge, 2001). *Helicobacter pylori* infection plays a major role in the development of chronic atrophic gastritis, the first step of the cascade (Genta and Rugge, 2001). The TGFB pathway occupies a central position among signaling networks that control growth, differentiation, apoptosis, and extracellular matrix accumulation of a wide variety of cell types, as well as the immune system. Despite its potent growth inhibition in normal epithelial and lymphoid cells, it is evident that TGFB signaling may act either as a suppressor or a promoter during carcinogenesis (Kim et al., 2000; Elliott and Blobe, 2005). However, a systematic analysis of the expressional profile of the TGFB signaling pathway components during the sequence of changes leading to carcinogenesis has not been conducted.

TGFB is a member of a large family of disulfide-bonded cytokines. Binding of TGFB to the TGFR2-TGFR1 (ALK5) heterodimer leads to phosphorylation of TGFR1. TGFR1 activation results in phosphorylation of particular SMAD proteins, which form heteromeric complexes and translocate to the nucleus, where they may direct transcriptional responses (Derynck and Zhang, 1996; Massague, 1996, 1998). SMAD2 and 3 serve as receptor-regulated SMADs (R-SMADs) transducing TGFB/ACTIVIN-like signals, and SMAD1, 5, and 8 act as R-SMADs, transducing BMP-like signals, while SMAD4 is the only common-partner SMAD (Co-SMAD) in mammals, and is shared by the TGFB/ACTIVIN and BMP pathways. SMAD6 and SMAD7 function as inhibitory SMADs (I-SMADs) in

Abbreviations: TGFB, transforming growth factor-ß; TBRI, transforming growth factor-ß receptor I; TBRII, transforming growth factor-ß receptor II

TGFB/ACTIVIN and BMP signaling. However, accumulating evidence suggests that TGFB/ACTIVIN also signal through other pathways, such as MAPK and NF-κB (de Guise et al., 2006) and that TP53 and NF-κB directly affect SMAD-dependent TGFB signaling (Cordenonsi et al., 2003, 2007). In the nucleus, R-SMAD-SMAD4 heterodimers interact with various transcription factors and transcriptional coactivators or corepressors, leading to transcriptional regulation of target genes such as CDKN1A ($p21^{CIP1}$), CDKN1B ($p27^{KIP1}$), CDKN2B ($p15I^{NK4B}$), MYC, and CDC25A.

The expression of TGFB signaling proteins and its clinical significance in GC have been examined in several studies. Increased expression of TGFB has been reported in GC (Naef et al., 1997; Gold, 1999), whereas reduced expression of SMAD3 and 4 was recently reported to be correlated with unfavorable clinical parameters in GC (Han et al., 2004; Kim et al., 2004; Wang et al., 2007). Overexpression of TP53 was also correlated with a poor prognosis for patients (Okuyama et al., 2002). However, these studies, for the most part, examined a single component of the TGFB pathway and, thus, there is some limitation in extrapolating the meaning of these results to the entire TGFB signaling pathway. Furthermore, the expression of TGFB signaling proteins and their downstream target proteins in gastric pre-cancerous lesions such as dysplasia have rarely been studied, with the exception of CDKN1A, so it is difficult to infer the potential roles during the early stages of tumorigenesis in the stomach. Therefore, a holistic and systematic study of the expression of TGFB signaling proteins along the dysplasia-carcinoma sequence of gastric neoplasias is required.

In this study, we employed tissue microarray-based immunohistochemical analysis as a large-scale screening technique and studied the expression of all the TGFB signaling proteins in GC and related lesions to provide insights into potential therapeutic interventions that could be used to prevent and treat GC by modulating the TGFB signaling pathway.

Materials and methods

Patients, Samples and Tissue Microarrays

Tissues from patients with GC and other gastric lesions, including 112 GCs, 20 high grade dysplasias (HDs), 17 low grade dysplasias (LDs), 54 intestinal metaplasias (IMs), 29 chronic atrophic gastritis (CAG) samples, and 54 normal gastric epithelium (NL) samples, were used for tissue microarray construction as previously described (Wang et al., 2007). This study was reviewed and approved by the ethics committee of the institutional review board of Chungbuk National University Hospital. The 112 gastric carcinomas [age = 49-85 years; average age = 66.1 years; 40 female and 72 male cases] were comprised of 30 early cases (pTis=1, pT1=29) and 82 advanced cases (pT2=26, pT3=42, pT4=14). A total of 25, 41, and 29 cases (26.5, 41.8, and 31.7%, respectively) were classified as well, moderately, and poorly differentiated adenocarcinomas, respectively. Histologically, 64 cases of GCs were intestinal type and 31 cases of GCs were diffuse type. However, in all cases, IM or dysplasia was accompanied. All archival materials were routinely fixed in 10% neutral-buffered formalin and embedded in paraffin. These samples were used to generate tissue microarray slides (3 mm in diameter) according to the standard method previously described (Choi et al., 2007; Wang et al., 2007).

Immunohistochemistry

The selection of the thirteen antibodies used in this study was based on the known components of the TGFB signaling pathway (Fig. 1), as well as the availability and suitability of the antibody for paraffin-embedded archival materials (Table 1). The components examined included TGFB signaling proteins [TGFB1/2, TGFBR1 (ALK5), TGFBR2, SMAD1/2/3, SMAD2/3, SMAD4, SMAD7] and downstream targets of TGFB-regulated transcription [CDKN1A ($p21^{CIP1}$), CDKN1B ($p27^{KIP1}$), MYC, CDC25A], as well as RELA (p65NF-κB) and TP53. Once the antibodies were selected, the specificity of the primary antibodies used in these experiments was confirmed by Western blotting of nine human GC cell lines, SNU5, SNU16, SNU216, SNU484, SNU638, MKN28, MKN74, KATOIII, and AGS (data not shown). Each antibody was then titrated with three to five different dilutions (with a 2-fold difference between each dilution) on whole-mount tissue sections, according to the manufacturer's recommendations. If there were no well-established positive control tissues, we used our in-house multi-tumor array to find a positive-control tissue. If signal-to-background ratio was not acceptable for the dilution tested, the antigen retrieval buffer, blocking solution, incubation time, and antibody concentration were readjusted. The dilutions of primary antibody and the antigen retrieval buffer used for each antibody are listed in Table 1. Dextran polymer conjugated goat anti-mouse, or goat anti-rabbit antibodies and horseradish peroxidase (DAKO Glostrup Denmark, Envision+) were used to detect primary antibody binding. Immunostaining was performed as previously described (Wang et al., 2007).

Evaluation of results of immunohistochemical staining

The evaluation of both the intensity of immunohistochemical staining and the proportion of positively-stained epithelial cells was performed as previously described (Wang et al., 2007). The staining intensity was classified as: 1+, weak; 2+, moderate; or 3+, strong. The percentage of positively staining tumor cells relative to the total number of tumor cells was assigned to one of five ranges: 0, < 5%; 1, 5-25%; 2, 26-50%; 3, 51-75%; and 4, > 75%. The scores for percentage of positive tumor cells and the staining intensity were multiplied to produce a weighted

immunoreactive score (IS) for each tumor specimen. Each lesion was examined and scored separately by two pathologists, and cases with discrepant scores were reevaluated to achieve a consensus score. The expression of TGFB1/2, TGFBR1, TGFBR2, SMAD7, MYC, and RELA in the cytoplasm was analyzed, while the expression of SMAD1/2/3, SMAD2/3, and SMAD4 was separately analyzed in both cytoplasm and nucleus. The expression of CDKN1A, and CDKN1B, CDC25A, and TP53 was restricted to the nucleus (Fig. 2). In addition, the IS was classified into three groups: very weak (IS=0-1), moderate (2-5), and high (IS=6-12). Changes in expression profile for each antigen were also analyzed.

Statistical analysis

Statistical analyses were performed using the Fisher's exact test, Pearson's χ^2 test, Mann-Whitney test, Kruskal-Wallis test, and the Tukey's HSD and Duncan's tests as post hoc tests. For comparison of means of ISs, the Mann-Whitney test, Kruskal-Wallis test, Tukey's HSD, and Duncan's test were used. The association of the expression level with clinicopathological factors was assessed by cross-tabulation, and significant differences were determined by the Fisher's exact test and Pearson's χ^2 test. A P value less than 0.05 was regarded as statistically significant. All statistical analyses were performed using SPSS software. (SPSS, Chicago, USA).

Results

The expression profiles of TGFB pathway signaling molecules and TP53 in GC and related lesions

The mean IS of each molecule in GC and related lesions, including CAG, IM, and dysplasia are illustrated in Fig. 3 (for detail see Table 2). Changes in expression profile were also similar when the staining levels were grouped as very weak, moderate, and high intensity staining (Table 3). These dynamic expression modes can be categorized by three patterns (Fig. 4A). In the first pattern, TGFB1/2, TGFBR1, MYC and TP53 showed a continuous increase in expression along the NL CAG-dysplasia-GC sequence (Fig. 4A). All these molecules were significantly enhanced in GC (Tables 2, 3). In addition, TGFB and TGFBR1 expression was

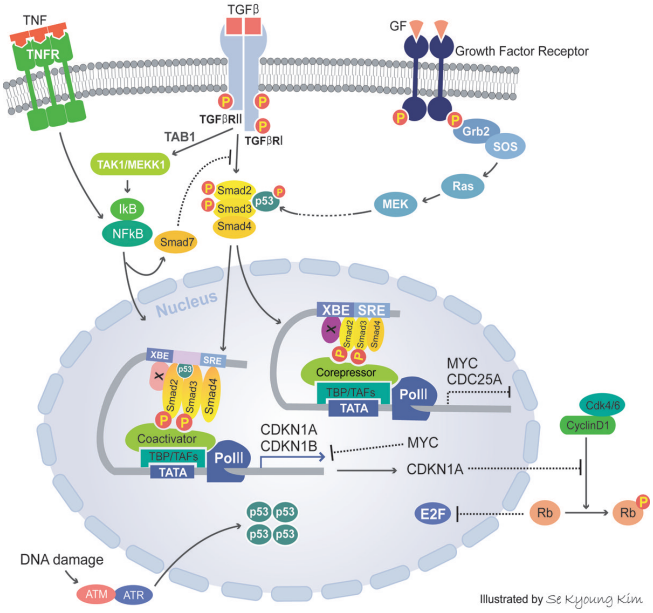

Fig. 1. Schematic drawing of the TGFB signaling pathway.

Table 1. Primary antibodies and antigen retrieval buffers used in this study.

Antibody	Type	Source	Catologue No.	Dilution	Antigen retrieval buffer
TGFB1/2	Poly (R)	S-cruz	sc146	1:40	Borate-EDTA
TGFBR1 (ALK5)	Poly (R)	S-cruz	sc398	1:40	Borate-EDTA
TGFBR2	E-6 (IgG2a)	S-cruz	sc17792	1:40	Borate-EDTA
SMAD2/3*	Poly (G)	S-cruz	sc6032	1:40	Tris-EDTA
SMAD1/2/3	H-2 (IgG2a)	S-cruz	sc7960	1:50	Borate-EDTA
SMAD4	B-8 (IgG1)	S-cruz	sc7966	1:50	Borate-EDTA
SMAD7	Poly(R)	S-cruz	sc11392	1:30	Tris-EDTA
CDKN1A ($p21^{CIP1}$)	HZ52 (IgG1)	Lab vision	MS387	1:40	Borate-EDTA
CDKN1B ($p27^{KIP1}$)	F-8 (IgG1)	S-cruz	sc1641	1:30	Borate-EDTA
TP53	DO7 (IgG2b)	Novocastra	NCL-p53-DO7	1:50	Citrate
RELA (p65NF-κB)	F-6 (IgG1)	S-cruz	sc8008	1:50	Tris-EDTA
MYC	9E11 (IgG1)	DiNonA	55720E	1:50	Citrate
CDC25A	DCS120+/ DCS121	NeoMarker	CDC25Ab-3	1:20	Borate-EDTA

Abbreviation: Poly(R), rabbit polyclonal, Poly(G), goat polyclonal, S-cruz, Santa Cruz Biotechnology, Borate-EDTA : 50 mM Borate (pH 8.2) supplemented with 1 mM EDTA and 1 mM NaCl, Tris-EDTA : 40 mM Tris (pH 9.5) supplemented with 1 mM EDTA, Citrate : 10 mM sodium citrate (pH 6.0), SMAD2/3* : Rabbit anti-goat Antibody (Vector Labs, 1:250) was introduced after primary antibody incubation. Then Envision+ kit (anti-rabbit) was applied.

significantly elevated in dysplasia. In the second pattern, tumor suppressor proteins such as SMAD1/2/3, SMAD2/3, SMAD4, CDKN1A, and CDKN1B showed an inverse V-shaped pattern with a peak in dysplasia, which continuously increased from NL to dysplasia, but decreased in GC (Fig. 4A). Expression of these molecules was significantly enhanced in dysplasia (Tables 2, 3). In the third pattern, TGFBR2, SMAD7, RELA, and CDC25A expression followed a sigmoid curve type with a plateau from dysplasia to GC (Fig. 4A). These proteins were upregulated in neoplastic lesions, including dysplasia and GC, but an additional increase in GC was not observed (Tables 2, 3).

Continuously increasing type: TGFB1/2, TGFBR1, MYC and TP53

In normal and non-neoplastic epithelia (CAG and IM), weak immunoreactivity for TGFB was rarely found in the cytoplasm of the mucosal epithelial cells, particularly in the mucous neck region. However, TGFB immunoreactivity was significantly enhanced in LD (IS: LD Vs NL, CAG $P < 0.01$ respectively) and reached its highest level in GC (IS: CA Vs HD $P < 0.025$, CA Vs LD $P < 0.001$, CA Vs non-neoplastic lesions $P < 0.001$). The overall expression pattern of TGFBR1 is similar to that of TGFB. Immunoreactivity for TGFBR1 was also rare and weak in non-neoplastic lesions, and was also significantly elevated in dysplasia (IS: LD, HD Vs non-neoplastic epithelia $P < 0.005$ respectively). Expression of TGFBR1 was most enhanced in carcinoma (IS: CA Vs LD, HD $P < 0.025$, CA Vs non-neoplastic lesions $P < 0.001$).

Immunoreactivities for MYC and TP53 were characteristically strong in GC compared to any other precursor lesions (IS: CA Vs all other lesions $P < 0.01$ in both MYC and TP53), whereas the expression levels of these proteins were low and similar to each other in all non-carcinoma lesions.

Inverse V-shaped type: SMAD1/2/3, SMAD2/3, SMAD4, CDKN1A, CDKN1B

Cytoplasmic SMAD1/2/3 (SMAD1/2/3(C)) immunoreactivity was very rare and weak in NL, but its level in preneoplastic lesions, including CAG and IM were more elevated compared to NL (IS: NL Vs CAG $P < 0.005$, NL Vs IM $P < 0.001$). SMAD1/2/3(C) levels were most highly enhanced in LD, but then were significantly reduced in HD and GC (IS: LD Vs any other lesions $P < 0.005$). The overall expression pattern of cytoplasmic SMAD2/3 (SMAD2/3(C)) was very similar to that of SMAD1/2/3. However, the highest expression of SMAD2/3(C) was observed in HD, not in LD, and significant down-regulation in GC was disclosed (IS: HD Vs CA $P < 0.05$) The overall expression pattern of cytoplasmic SMAD4 (SMAD4(C)) was also similar to SMAD1/2/3(C) and SMAD2/3(C), with minor differences. Its level was highest in LD and abruptly reduced in GC (IS: LD, HD Vs CA $P < 0.05$). In contrast to the cytoplasmic expression of SMAD1/2/3, the immunoreactivity of nuclear SMAD1/2/3 (SMAD1/2/3(N)) was frequently observed in NL, and there was no significant difference in expressional level among all lesions. However, the nuclear staining of SMAD2/3 (SMAD 2/3(N)) was strongest in HD and also significantly decreased in GC like SMAD2/3(C) (IS: HD Vs all other lesions $P < 0.01$). The expression pattern in nuclear SMAD4 (SMAD4(N)) was similar to SMAD4(C) pattern and the highest expression was noted in dysplasia, but fell dramatically in carcinoma, and was reduced to the level of non-neoplastic lesions (IS: LD Vs CA $P < 0.005$, HD Vs CA $P < 0.001$).

CDKN1A (p21) was expressed weakly in the nucleus of epithelial cells of normal gastric mucosa and its expression level was enhanced in non-neoplastic inflammatory lesions, including CAG and IM (IS: NL Vs CAG, IM $P < 0.001$). The strongest immunoreactivity was observed in the nucleus of tumor cells of both low and high grade dysplasia (IS: LD, HD Vs NL, CAG, IM $P < 0.001$ in all combinations). However, levels were reduced in carcinoma (IS: CA Vs LD, HD $P < 0.001$). CDKN1B (p27) was also expressed in the nucleus of various cells, and levels in normal and non-neoplastic lesions were very low. Similar to other tumor suppressor proteins, levels of CDKN1B (p27) were greatly increased in dysplasia (IS: LD, HD Vs NL, CAG, IM $P < 0.001$ in all combinations). The level was also markedly down-regulated in carcinoma (IS: CA Vs LD, HD $P < 0.001$).

Table 2. The immunoreactivity scores of TGFB signaling components in carcinoma, dysplasia, metaplasia, chronic atrophic gastritis and normal epithelium of the stomach.

	TGFB 1/2	TGFBR1 (ALK5)	TGFBR2	SMAD 1/2/3 Cyt	SMAD 1/2/3 Nu	SMAD 2/3 Cyt	SMAD 2/3 Nu	SMAD 4 Cyt	SMAD 4 Nu	SMAD7	CDKN1A (p21)	CDKN1B (p27)	MYC	CDC25A	REL A (p65NFKB)	TP53
CA	4.04±2.44	5.23±2.93	2.89±1.88	3.27±2.21	1.72±1.98	2.68±1.89	2.32±2.12	2.46±2.12	2.12±2.18	4.72±2.82	5.10±3.29	1.49±1.74	4.90±2.71	4.99±2.75	5.85±3.25	2.67±3.89
AD (HD)	2.20±1.15	4.00±2.22	2.10± 1.37	3.10±1.92	2.00±1.75	4.33±2.14	3.83±2.31	4.60±1.31	3.55±1.70	4.45±2.39	8.40±3.69	3.85±2.96	2.39±1.58	4.94±3.30	4.89±2.21	0.84±2.85
AD (LD)	2.76±1.39	3.88±1.83	3.12± 1.22	4.76±1.09	1.82±1.59	2.21±1.67	1.71±1.44	5.59±2.53	3.47±1.84	5.53±1.50	8.29±3.18	4.00±2.88	3.08±2.60	5.64±1.78	5.53±2.00	0.15±0.55
IM	1.71±1.55	2.31±1.45	1.46± 1.16	2.29±1.50	1.45±1.29	2.00±1.48	2.88±1.35	2.86±1.57	2.49±1.43	3.30±2.27	3.45±2.49	0.95±0.80	3.18±1.77	3.16±2.05	2.71±1.58	0.00±0.00
CAG	1.04±1.18	1.67±1.52	1.68± 1.36	2.03±1.70	2.03±1.21	1.13±1.15	2.38±1.17	2.00±1.86	2.79±1.62	2.52±2.16	4.35±2.39	0.80±0.71	2.54±1.84	3.61±2.11	1.72±1.57	0.00±0.00
NL	1.31±1.85	0.89±0.98	0.96± 1.16	0.86±1.02	1.80±1.30	0.15±0.44	2.12±1.23	0.49±0.78	1.57±1.38	0.92±1.05	1.40±1.81	0.13±0.33	2.08±1.86	3.08±1.86	0.85±0.92	0.00±0.00

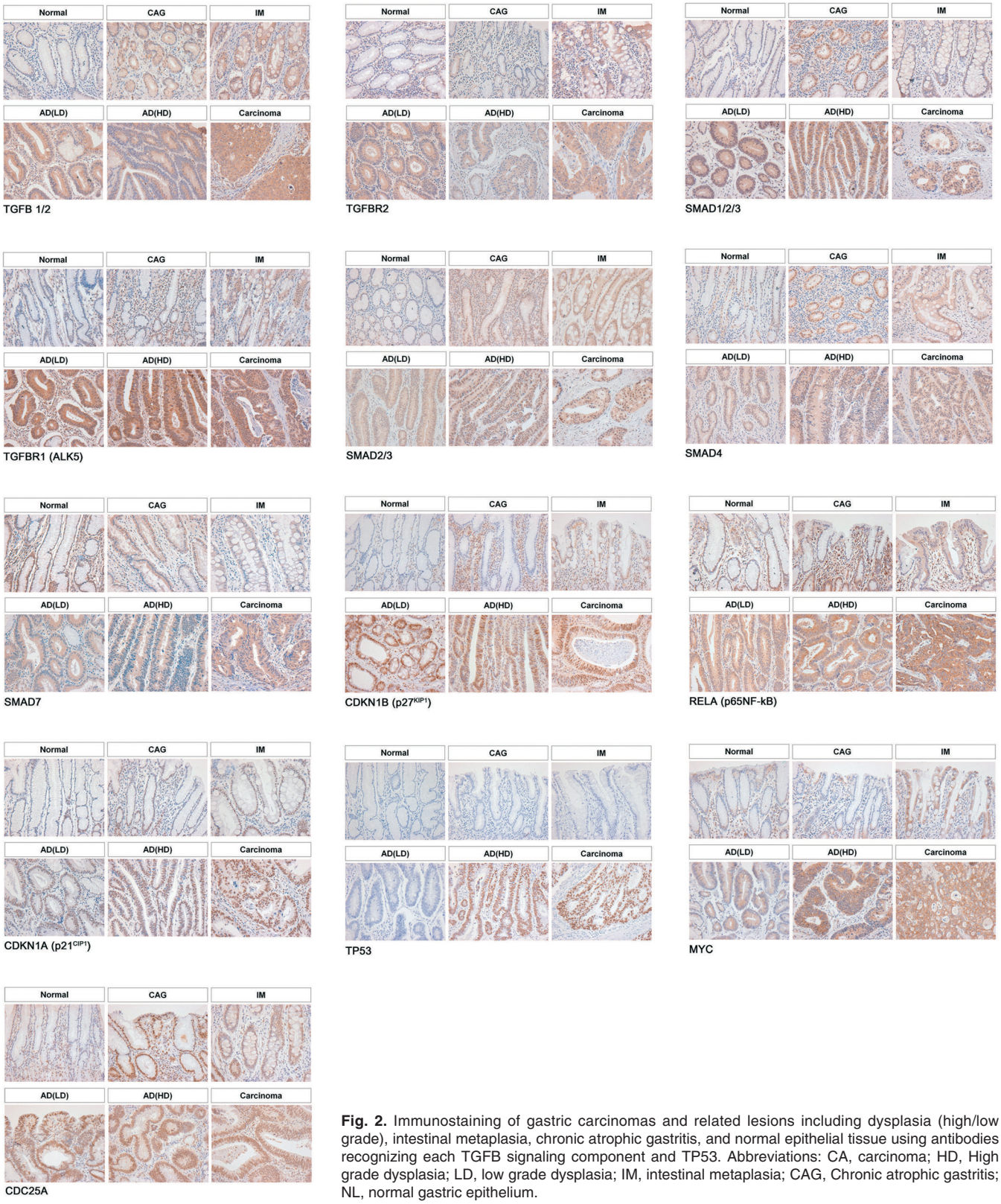

Fig. 2. Immunostaining of gastric carcinomas and related lesions including dysplasia (high/low grade), intestinal metaplasia, chronic atrophic gastritis, and normal epithelial tissue using antibodies recognizing each TGFB signaling component and TP53. Abbreviations: CA, carcinoma; HD, High grade dysplasia; LD, low grade dysplasia; IM, intestinal metaplasia; CAG, Chronic atrophic gastritis; NL, normal gastric epithelium.

Table 3. The expression profiles of TGFB signaling components and TP53 in gastric carcinomas and related precursor lesions.

		NL	CAG	IM	LD	HD	CA	P-value
TGFB1/2	W*	35/52 (67.3%)	18/26 (69.2%)	20/42 (47.6%)	2/17 (11.8%)	4/20 (20.0%)	20/110 (18.2%)	P<0.001 (LD vs IM: P<0.001, CA vs HD: P<0.025)
	M**	14/52 (26.9%)	8/26 (30.8%)	21/42 (50.0%)	15/17 (88.2%)	16/20 (80.0%)	57/110 (51.8%)	
	S***	3/52 (5.8%)	0/26 (0.0%)	1/42 (2.4%)	0/17 (0.0%)	0/20 (0.0%)	33/110 (30.0%)	
TGFBR1	W	42/54 (77.8%)	16/27 (59.3%)	16/51 (31.4%)	2/17 (11.8%)	4/20 (20.0%)	16/109 (14.7%)	P<0.001 (IM vs CAG: P<0.025, LD vs IM: P<0.001, CA vs LD: P<0.025)
	M	12/54 (22.2%)	10/27 (37.0%)	35/51 (68.6%)	12/17 (70.6%)	11/20 (55.0%)	39/109 (35.8%)	
	S	0/54 (0.0%)	1/27 (3.7%)	0/51 (0.0%)	3/17 (17.6%)	5/20 (25.0%)	54/109 (49.5%)	
TGFBR2	W	39/53 (73.6%)	13/28 (46.4%)	33/54 (61.1%)	1/17 (5.9%)	9/20 (45.0%)	31/109 (28.4%)	P<0.001 (CAG vs NL: P<0.025, LD vs IM: P<0.001, HD vs LD: P<0.025)
	M	14/53 (26.4%)	13/28 (46.4%)	21/54 (38.9%)	15/17 (88.2%)	11/20 (55.0%)	55/109 (50.5%)	
	S	0/53 (0.0%)	2/28 (7.1%)	0/54 (0.0%)	1/17 (5.9%)	0/20 (0.0%)	23/109 (21.1%)	
SMAD1/2/3 (Cytoplasm)	W	41/51 (80.4%)	12/29 (41.4%)	21/55 (38.2%)	0/17 (0.0%)	5/20 (25.0%)	30/111 (27.0%)	P<0.001 (CAG vs NL: P<0.001, LD vs IM: P<0.001, CA vs LD: P<0.05)
	M	10/51 (19.6%)	15/29 (51.7%)	33/55 (60.0%)	14/17 (82.4%)	14/20 (70.0%)	68/111 (61.3%)	
	S	0/51 (0.0%)	2/29 (6.9%)	1/55 (1.8%)	3/17 (17.6%)	1/20 (5.0%)	13/111 (11.7%)	
SMAD1/2/3 (Nucleus)	W	23/51 (45.1%)	8/29 (27.6%)	32/55 (58.2%)	10/17 (58.8%)	9/20 (45.0%)	62/111 (55.9%)	P<0.05 (IM vs CAG: P<0.01)
	M	28/51 (54.9%)	21/29 (72.4%)	23/55 (41.8%)	7/17 (41.2%)	11/20 (55.0%)	44/111 (39.6%)	
	S	0/51 (0.0%)	0/29 (0.0%)	0/55 (0.0%)	0/17 (0.0%)	0/20 (0.0%)	5/111 (4.5%)	
SMAD2/3 (Cytoplasm)	W	33/34 (97.1%)	18/24 (75.0%)	16/41 (39.0%)	5/14 (35.7%)	3/18 (16.7%)	34/109 (31.2%)	P<0.001 (CAG vs NL: P<0.025, IM vs CAG: P<0.01, HD vs LD: P<0.025, CA vs HD: P<0.01)
	M	1/34 (2.9%)	6/24 (25.0%)	25/41 (61.0%)	9/14 (64.3%)	9/18 (50.0%)	68/109 (62.4%)	
	S	0/34 (0.0%)	0/24 (0.0%)	0/41 (0.0%)	0/14 (0.0%)	6/18 (33.3%)	7/109 (6.4%)	
SMAD2/3 (Nucleus)	W	10/34 (29.4%)	6/24 (25.0%)	5/41 (12.2%)	6/14 (42.9%)	3/18 (16.7%)	46/109 (42.2%)	P<0.001 (LD vs IM: P<0.05, CA vs IM: P<0.001)
	M	24/34 (70.6%)	18/24 (75.0%)	34/41 (82.9%)	8/14 (57.1%)	11/18 (61.1%)	53/109 (48.6%)	
	S	0/34 (0.0%)	0/24 (0.0%)	2/41 (4.9%)	0/14 (0.0%)	4/18 (22.2%)	10/109 (9.2%)	
SMAD4 (Cytoplasm)	W	46/53 (86.8%)	13/27 (48.1%)	11/49 (22.4%)	1/17 (5.9%)	0/20 (0.0%)	42/112 (37.5%)	P<0.001 (CAG vs NL: P<0.001, LD vs IM: P<0.001, HD vs LD: P<0.05, CA vs HD: P<0.001)
	M	7/53 (13.2%)	14/27 (51.9%)	37/49 (75.5%)	9/17 (52.9%)	18/20 (90.0%)	64/112 (57.1%)	
	S	0/53 (0.0%)	0/27 (0.0%)	1/49 (2.0%)	7/17 (41.2%)	2/20 (10.0%)	6/112 (5.4%)	
SMAD4 (Nucleus)	W	26/53 (49.1%)	6/28 (21.4%)	12/49 (24.5%)	3/17 (17.6%)	2/20 (10.0%)	57/112 (50.9%)	P<0.001 (CAG vs NL: P<0.025, CA vs HD: P<0.01)
	M	27/53 (50.9%)	20/28 (71.4%)	36/49 (73.5%)	12/17 (70.6%)	14/20 (70.0%)	48/112 (42.9%)	
	S	0/53 (0.0%)	2/28 (7.1%)	1/49 (2.0%)	2/17 (11.8%)	4/20 (20.0%)	7/112 (6.3%)	
SMAD7	W	40/48 (83.3%)	10/25 (40.0%)	12/50 (24.0%)	0/17 (0.0%)	1/20 (5.0%)	15/110 (13.6%)	P<0.001 (CAG vs NL: P<0.001, LD vs IM: P<0.001)
	M	8/48 (16.7%)	12/25 (48.0%)	29/50 (58.0%)	8/17 (47.1%)	11/20 (55.0%)	54/110 (49.1%)	
	S	0/48 (0.0%)	3/25 (12.0%)	9/50 (18.0%)	9/17 (52.9%)	8/20 (40.0%)	41/110 (37.3%)	
CDKN1A	W	35/53 (66.0%)	1/23 (4.3%)	10/47 (21.3%)	0/17 (0.0%)	1/20 (5.0%)	22/109 (20.2%)	P<0.001 (CAG vs NL: P<0.001, LD vs IM: P<0.001, CA vs HD: P<0.05)
	M	15/53 (28.3%)	16/23 (69.6%)	28/47 (59.6%)	3/17 (17.6%)	4/20 (20.0%)	39/109 (35.8%)	
	S	3/53 (5.7%)	6/23 (26.1%)	9/47 (19.1%)	14/17 (82.4%)	15/20 (75.0%)	48/109 (44.0%)	
CDKN1B	W	48/48 (100.0%)	21/25 (84.0%)	31/41 (75.6%)	3/14 (21.4%)	7/20 (35.0%)	66/110 (60.0%)	P<0.001 (CAG vs NL: P<0.01, LD vs IM: P<0.001, CA vs HD: P<0.001)
	M	0/48 (0.0%)	4/25 (16.0%)	10/41 (24.4%)	7/14 (50.0%)	6/20 (30.0%)	41/110 (37.3%)	
	S	0/48 (0.0%)	0/25 (0.0%)	0/41 (0.0%)	4/14 (28.6%)	7/20 (35.0%)	3/110 (2.7%)	
MYC	W	21/39 (53.8%)	9/24 (37.5%)	8/38 (21.1%)	4/13 (30.8%)	7/18 (38.9%)	12/111 (10.8%)	P<0.001 (CA vs HD: P<0.01)
	M	14/39 (35.9%)	14/24 (58.3%)	26/38 (68.4%)	6/13 (46.2%)	10/18 (55.6%)	54/111 (48.6%)	
	S	4/39 (10.3%)	1/24 (4.2%)	4/38 (10.5%)	3/13 (23.1%)	1/18 (5.6%)	45/111 (40.5%)	
CDC25AC (Nucleus)	W	9/39 (23.1%)	4/28 (14.3%)	8/43 (18.6%)	0/14 (0.0%)	1/18 (5.6%)	8/108 (7.4%)	P<0.001 (LD vs IM: P<0.01)
	M	27/39 (69.2%)	19/28 (67.9%)	29/43 (67.4%)	6/14 (42.9%)	10/18 (55.6%)	56/108 (51.9%)	
	S	3/39 (7.7%)	5/28 (17.9%)	6/43 (14.0%)	8/14 (57.1%)	7/18 (38.9%)	44/108 (40.7%)	
RELA	W	36/46 (78.3%)	15/25 (60.0%)	9/41 (22.0%)	0/17 (0.0%)	1/19 (5.3%)	15/110 (13.6%)	P<0.001 (IM vs CAG: p<0.01, LD vs IM: P<0.01)
	M	10/46 (21.7%)	8/25 (32.0%)	28/41 (68.3%)	9/17 (52.9%)	9/19 (47.4%)	35/110 (31.8%)	
	S	0/46 (0.0%)	2/25 (8.0%)	4/41 (9.8%)	8/17 (47.1%)	9/19 (47.4%)	60/110 (54.5%)	
TP53	W	62/62 (100.0%)	26/26 (100.0%)	54/54 (100.0%)	12/13 (92.3%)	17/19 (89.5%)	69/111 (62.2%)	P<0.005 (CA vs IM/CAG/NL: P<0.001)
	M	0/62 (0.0%)	0/26 (0.0%)	0/54 (0.0%)	1/13 (7.7%)	1/19 (5.3%)	13/111 (11.7%)	
	S	0/62 (0.0%)	0/26 (0.0%)	0/54 (0.0%)	0/13 (0.0%)	1/19 (5.3%)	29/111 (26.1%)	

*Very weak (W): Immunoreactive Score (IS) = 0-1; **Moderate (M): IS = 2-5; ***Strong (S): IS = 6-12. Abbreviations: CA, Carcinoma; HD, high grade dysplasia; LD, Low grade dysplasia; IM, Intestinal metaplasia; CAG, Chronic atrophic gastritis; NL, Normal.

Sigmoid curve type: TGFBR2, SMAD7, RELA, CDC25A

Immunoreactivity for TGFBR2 was weak in non-neoplastic epithelium, especially in normal epithelium (IS: NL Vs CAG $P < 0.05$), but the level was significantly enhanced in dysplasia and carcinoma (IS: LD, CA Vs any non-neoplastic epithelium $P < 0.005$). However, in contrast to TGFB, TGFBR1, MYC, and TP53, the level of TGFBR2 expression appeared to plateau in dysplasia and did not increase further in carcinoma. Similar expression patterns were observed for SMAD7, RELA (NF-κB), CDC25A.

Clinicopathological correlation of the expression of TGFB signaling proteins and TP53 in GC

The correlation of TGFB signaling protein and TP53 expression in gastric adenocarcinomas with various clinicopathological parameters was analyzed. TGFB and MYC were significantly correlated with advanced

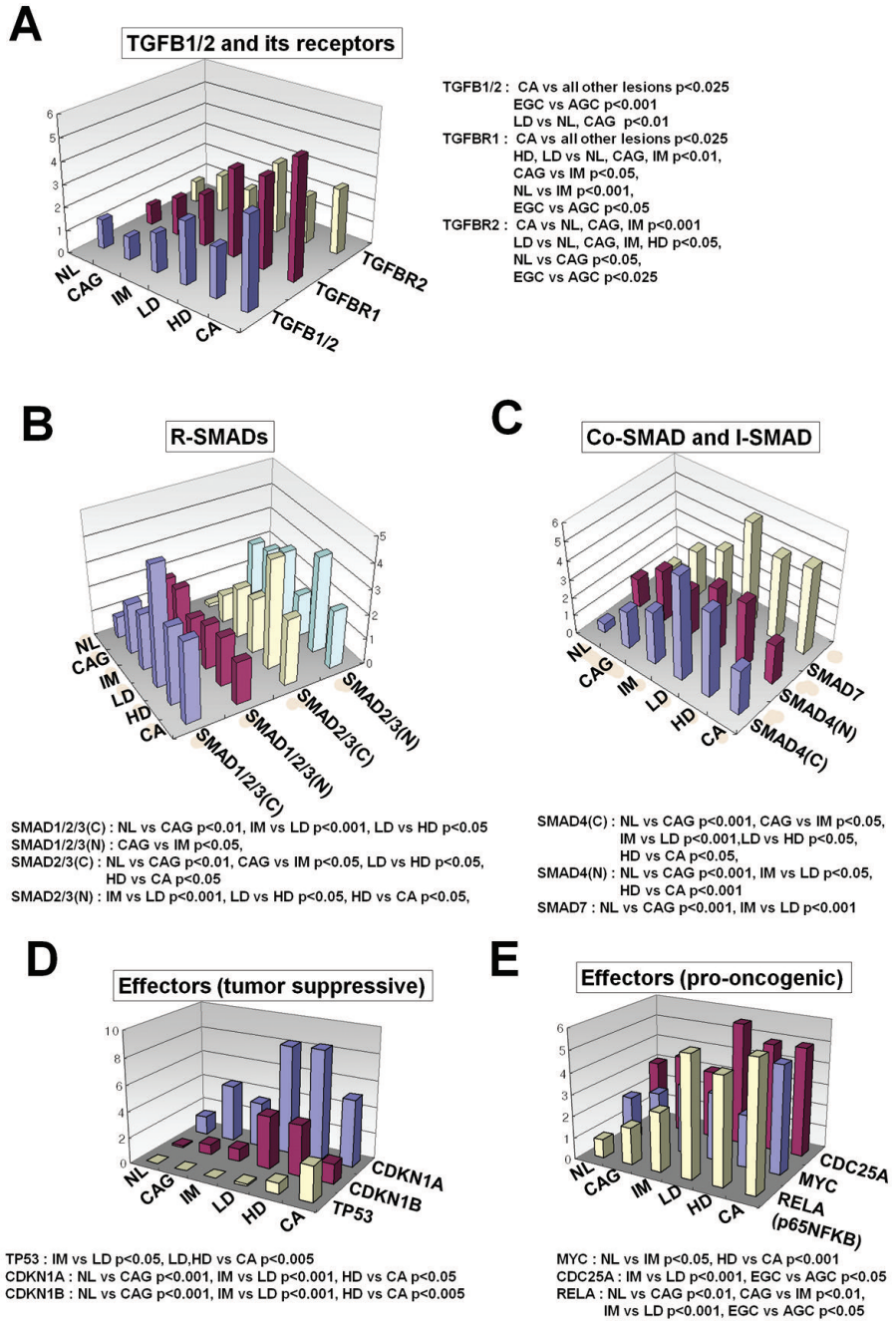

Fig. 3. The expression profile of each TGFB pathway component and TP53 in tissue samples of gastric cancers and related lesions. The mean immunoreactive score (IS) for each component in each type of lesion is shown graphically. The TGFB pathway components can be divided into TGFB and its receptors **(A)**, R-SMADs including SMAD1/2/3 and SMAD2/3 **(B)**, Co-SMADs and I-SMADs, such as SMAD4 and SMAD7 (**C**), tumor suppressive effectors, including CDKN1A, CDKN1B and TP53 **(D)**, pro-oncogenic effectors, including MYC, CDC25A, RELA **(E)**. Abbreviations: CA, carcinoma; HD, High grade dysplasia; LD, low grade dysplasia; IM, intestinal metaplasia; CAG, chronic atrophic gastritis; NL, normal gastric epithelium.

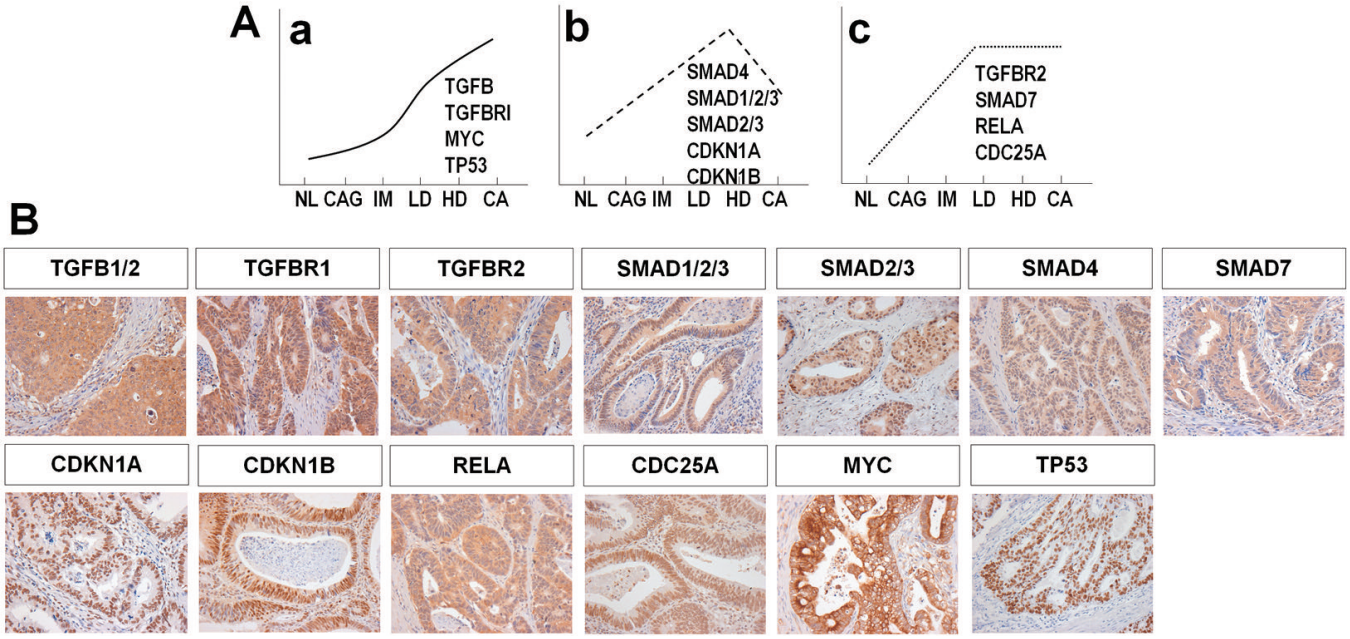

Fig. 4. A. Three types of expression patterns were observed for TGFB signaling molecules: consistently increasing (a), inverse V-shaped (b), and sigmoid curve (c). **B.** Immunostaining of gastric carcinoma tissues using antibodies which recognize each of the TGFB signaling molecules and TP53. Prominent cytoplasmic staining (TGFB, TGFBR1, TGFBR2, SMAD7, RELA and MYC), or nuclear staining (CDKN1A and TP53), or both cytoplasmic and nuclear staining (SMAD1/2/3, SMAD2/3, SMAD4, CDKN1B and CDC25A) were observed. Abbreviations: CA, carcinoma; HD, high grade dysplasia; LD, low grade dysplasia; IM, intestinal metaplasia; CAG, chronic atrophic gastritis; NL, normal gastric epithelium.

Table 4. Correlation of clinicopathologic parameters and immunoreactivity scores of TGF pathway components in gastric carcinoma.

	TGFB1/2	TGFBR1	TGFBR2	SMAD1/2/3 (C)	SMAD1/2/3 (N)	SMAD2/3 (C)	SMAD2/3 (N)	SMAD4 (C)	SMAD4 (N)	SMAD7	CDKN1A	CDKN1B	MYC	CDC25A	RELA	TP53
Age (No.)	N.S.	P=0.06	P=0.10	P=0.15	N.S	N.S	N.S	N.S	N.S	P<0.05	P<0.05	P<0.05	N.S	N.S	N.S	P<0.05
≤ 55 years (32)	3.87±2.28	4.31±2.87	2.40±1.61	2.78±2.06	1.56±1.85	2.27±1.78	2.37±2.13	2.09±1.99	1.91±1.86	3.84±2.67	4.06±2.95	0.94±1.29	4.42±3.11	4.63±2.63	5.48±3.33	1.42±3.16
> 55years (79)	4.10±2.53	5.53±2.91	3.08±1.95	3.44±2.26	1.78±2.05	2.84±1.92	2.30±2.13	2.62±2.17	2.20±2.31	5.06±2.82	5.47±3.37	1.71±1.85	5.09±2.54	5.13±2.80	6.00±3.23	3.15±4.05
Sex (No.)	N.S.	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S
Female (40)	4.18±2.60	5.03±2.80	2.84±1.91	3.15±2.43	1.90±2.24	2.42±1.84	2.53±2.23	2.13±2.29	2.10±2.18	4.77±2.52	5.26±3.45	1.37±1.63	4.61±2.83	5.14±2.80	5.87±3.16	3.03±3.89
Male (72)	3.96±2.39	5.30±3.02	2.92±1.88	3.31±2.09	1.62±1.85	2.82±1.91	2.21±2.07	2.65±2.02	2.13±2.19	4.69±2.99	4.94±3.23	1.56±1.80	5.05±2.65	4.92±2.74	5.85±3.32	2.48±3.90
LN metastasis (No.)	P=0.06	N.S	N.S	N.S	P<0.05	N.S	N.S	P<0.01	P<0.01	N.S	N.S	N.S	P=0.127	P<0.05	N.S	N.S
Negative (41)	3.46±2.13	5.32±3.38	2.66±1.78	3.39±1.93	2.17±2.00	2.88±1.82	2.43±2.06	3.29±2.24	2.95±2.19	4.83±2.50	5.37±3.11	1.59±1.87	4.40±2.59	4.24±2.02	5.73±3.05	2.48±3.87
Positive (68)	4.38±2.60	5.13±2.67	3.00±1.94	3.14±2.38	1.48±1.96	2.56±1.95	2.29±2.17	1.96±1.91	1.63±2.05	4.67±3.02	4.76±3.33	1.46±1.67	5.22±2.77	5.38±3.01	5.90±3.40	2.82±3.94
Tumor size (No.)	N.S	P=0.12	N.S	N.S	P<0.01	N.S	N.S	P=0.17	P<0.01	P<0.05	N.S	N.S	P<0.05	P<0.05	P<0.05	N.S
≥ 5.0cm (65)	4.30±2.74	5.64±2.72	3.11±2.00	3.39±2.35	1.11±1.72	2.81±1.86	2.24±2.27	2.19±2.07	1.56±2.09	5.28±3.10	4.89±3.12	1.30±1.50	5.46±2.68	5.59±3.08	6.50±3.07	2.84±3.97
< 5.0cm (45)	3.76±2.10	4.76±3.10	2.67±1.75	3.11±2.08	2.35±2.07	2.55±1.92	2.40±1.98	2.75±2.16	2.69±2.14	4.11±2.36	5.22±3.49	1.69±1.95	4.33±2.65	4.39±2.24	5.19±3.32	2.49±3.83
Gross type (No.)	P<0.01	P<0.05	P<0.05	N.S	P<0.001	N.S	P<0.05	P<0.05	P<0.001	N.S	P=0.18	N.S	P<0.01	P<0.05	P=0.07	N.S
EGC (30)	2.90±1.83	4.33±3.30	2.27±1.78	2.87±1.87	3.13±2.11	2.97±1.99	2.81±1.80	3.23±1.96	3.80±2.12	4.17±2.28	5.73±3.51	1.48±1.41	3.52±2.69	4.07±2.10	4.93±2.86	2.10±3.52
AGC (82)	4.46±2.53	5.53±2.73	3.13±1.88	3.40±2.32	1.20±1.68	2.56±1.85	2.13±2.22	2.18±2.13	1.50±1.86	4.91±2.98	4.80±3.20	1.49±1.86	5.44±2.54	5.35±2.90	6.20±3.34	2.89±4.02
Histological Type (No.)	P<0.05	P<0.01	P<0.01	P<0.001	N.S	P<0.01	P<0.05	P<0.001	P=0.12	P<0.05	P=0.12	P<0.001	P<0.01	P<0.05	P<0.05	P<0.05
Intestinal (64)	4.69±2.34	6.19±2.61	3.55±1.66	4.08±1.92	1.69±2.06	3.31±1.77	2.62±2.33	3.17±2.15	2.28±2.35	5.52±2.70	5.68±3.25	2.06±1.89	5.78±2.31	5.78±2.75	6.67±2.78	3.49±4.22
Diffuse (31)	3.48±2.35	4.50±2.69	2.33±1.86	2.41±2.20	1.59±1.95	1.77±1.52	1.67±1.52	1.48±1.54	1.58±1.64	4.03±2.66	4.55±3.32	0.80±1.10	4.13±2.55	4.07±2.12	5.32±3.17	1.52±3.06
Grade (No.)	P<0.05	P<0.05	P<0.05	P<0.05	N.S	P<0.01	P=0.06	P<0.05	P<0.05	P<0.05	P<0.05	P<0.01	P<0.01	N.S	P<0.05	P=0.08
I (25)	4.60±2.31	6.48±2.96	3.32±1.60	4.20±1.85	2.00±1.71	3.81±1.58	2.96±2.14	4.16±1.57	3.40±2.04	5.28±2.81	7.13±2.51	2.65±2.08	4.88±1.88	5.56±2.60	6.44±2.75	2.92±4.19
II (41)	4.90±2.37	6.22±2.40	3.85±1.64	4.17±2.02	1.56±2.20	3.02±1.77	2.49±2.49	2.68±2.29	1.66±2.42	6.05±2.69	5.20±3.39	1.71±1.65	6.27±2.31	5.78±2.95	7.24±2.89	3.83±4.21
III (29)	3.31±2.33	4.32±2.54	2.07±1.68	2.17±1.91	1.60±2.01	1.82±1.68	1.57±1.50	1.35±1.36	1.52±1.67	3.63±2.20	4.03±3.01	0.79±1.10	4.48±2.89	4.37±1.90	5.14±3.10	1.90±3.34
Tumor invasion (No.)	P<0.05	P<0.05	P=0.10	P=0.10	P<0.001	P=0.08	N.S	P=0.07	P<0.001	P=0.08	P<0.05	N.S	P<0.01	P=0.09	P=0.08	N.S
I (29)	2.93±1.85	4.24±3.32	2.24±1.81	2.83±1.89	2.97±1.94	2.97±2.03	2.90±1.75	3.24±1.99	3.69±2.07	4.25±2.29	5.52±3.37	1.40±1.35	3.60±2.70	4.10±2.13	4.97±2.91	2.07±3.57
II (26)	4.46±2.40	6.32±2.79	3.36±1.50	3.62±2.17	2.15±2.22	3.08±1.35	2.00±2.47	2.41±2.42	1.74±2.12	4.81±2.50	5.84±3.40	1.80±2.57	4.92±2.62	4.80±2.20	6.44±3.10	3.38±4.45
III (42)	4.35±2.82	4.95±2.78	2.87±2.19	2.90±2.46	0.73±1.32	2.08±2.07	2.26±2.16	2.02±1.98	1.54±1.94	4.44±3.22	3.83±3.08	1.55±1.43	5.50±2.61	5.23±3.19	5.61±3.69	2.83±3.78
IV (14)	4.77±2.13	6.15±2.12	3.46±1.33	4.38±1.94	1.23±1.83	3.23±1.64	2.08±2.10	2.31±1.97	1.38±1.80	6.54±2.90	5.92±2.35	1.15±1.57	6.15±2.44	6.31±2.95	7.54±2.18	2.54±4.12
Stage (No.)	P<0.05	N.S	N.S	N.S	P<0.001	N.S	N.S	P<0.05	P<0.001	N.S	N.S	N.S	P<0.05	N.S	N.S	N.S
I (39)	3.21±2.04	4.77±3.35	2.49±1.75	3.10±1.94	2.74±2.11	3.00±1.84	2.70±1.99	3.28±2.26	3.38±2.21	4.34±2.28	5.77±3.46	1.67±1.96	3.95±2.68	4.26±2.02	5.33±3.02	2.13±3.67
II (10)	4.80±1.99	5.22±0.97	3.22±1.56	3.70±2.21	1.70±2.16	3.00±1.66	2.44±2.40	2.27±2.28	2.00±1.95	4.10±1.52	4.50±3.14	1.30±1.77	5.30±3.40	5.33±2.45	6.56±2.88	2.40±3.60
III (35)	4.69±2.74	5.66±3.06	3.11±2.00	3.28±2.54	0.83±1.40	2.47±1.96	2.09±2.37	2.11±1.86	1.47±2.05	4.94±3.27	4.62±3.18	1.60±1.63	5.40±2.63	5.03±2.95	5.94±3.79	2.97±4.11
IV (25)	4.12±2.57	5.24±2.60	3.00±2.02	3.20±2.24	1.48±1.85	2.36±2.00	2.08±1.89	1.72±1.90	1.12±1.51	5.24±3.29	4.48±2.97	1.20±1.55	5.60±2.33	5.79±3.28	6.20±2.97	3.32±4.15

clinical stages, whereas SMAD1/2/3(N), and SMAD4(C) and SMAD4(N), were correlated with early clinical stages (Table 4). TGFB, TGFBR1, and MYC were significantly enhanced in cases of deep tumor invasion, whereas SMAD1/2/3(N) and SMAD4(N) were elevated in cases of superficial invasion (Table 4). TGFB, TGFRI, TGFRII, MYC, and nuclear CDC25A were more highly expressed in AGC (advanced gastric cancer) than in EGC (early gastric cancer). In contrast, SMAD1/2/3(N) and SMAD2/3(N), and SMAD4(C) and SMAD4(N) were significantly upregulated in EGC (Table 4). CDC25A was positively correlated with nodal metastasis, while SMAD1/2/3(N), and SMAD4(C) and SMAD4(N) were inversely correlated with nodal metastasis (Table 4). The degree of SMAD7, MYC,

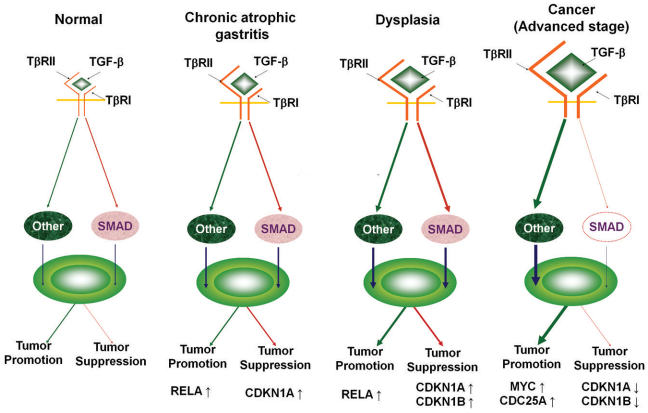

Fig. 5. Schematic presentation of a hypothesis for the effects of alterations in TGFB signaling during tumorigenesis.

	NL	CAG	IM	AD (LD)	AD (HD)	CA (early stage)	CA (advanced Stage)
TGFB1/2	+	+	+	++	++	++	++++
TGFBR1	±	+	++	+++	+++	+++	++++
TGFBR2	±	+	+	+++	++	++	+++
SMAD1/2/3(C)	±	++	++	++++	+++	++	+++
SMAD1/2/3(N)	+	++	+	+	++	+++	+
SMAD2/3(C)	±	+	++	++	++++	++	++
SMAD2/3(N)	++	++	++	+	+++	++	+
SMAD4(C)	±	+	++	++++	++++	+++	++
SMAD4(N)	+	++	++	+++	+++	+++	+
SMAD7	±	++	+++	++++	++++	++++	++++
CDKN1A	±	++	+	++++	++++	++	++
CDKN1B	-	±	±	++++	+++	+	+
MYC	++	++	++	++	++	+++	++++
CDC25A	+++	+++	+++	++++	+++	+++	++++
RELA	±	+	++	++++	++++	++++	++++
TP53	-	-	-	±	±	++	++

TGFB1/2 : IM vs LD P<0.05, EGC vs AGC P<0.005
TGFBR1 : NL vs CAG P<0.01, CAG vs IM p<0.05, IM vs LD P<0.005, EGC vs AGC P<0.05
TGFBR2 : NL vs CAG P<0.05, IM vs LD P<0.001, LD vs HD P<0.05, EGC vs AGC P<0.05
SMAD1/2/3(C) : NL vs CAG P<0.01, IM vs LD P<0.001, LD vs HD P<0.05
SMAD1/2/3(N) : CAG vs IM P<0.05, LD, HD vs EGC P<0.05, HD & EGC vs AGC P<0.001
SMAD2/3(C) : NL vs CAG P<0.01, CAG vs IM P<0.05, LD vs HD P<0.05, HD vs EGC P<0.05
SMAD2/3(N) : IM vs LD P<0.001, LD vs HD P<0.05, HD vs EGC P<0.05, EGC vs AGC P<0.05
SMAD4(C) : NL vs CAG P<0.001, CAG vs IM P<0.05, IM vs LD P<0.001, LD vs HD P<0.05, HD vs EGC P<0.05, EGC vs AGC P<0.05
SMAD4(N) : NL vs CAG P<0.001, IM vs LD P<0.05, EGC vs AGC P<0.001
SMAD7 : NL vs CAG P<0.001, IM vs LD P<0.001
CDKN1A : NL vs CAG P<0.001, IM vs LD P<0.001, HD vs EGC P<0.05
CDKN1B : NL vs CAG P<0.001, IM vs LD P<0.001, HD vs EGC P<0.005
MYC : EGC vs AGC P<0.001
CDC25A : IM vs LD P<0.001, EGC vs AGC P<0.05
RELA : NL vs CAG P<0.01, CAG vs IM P<0.01, IM vs LD P<0.001, EGC vs AGC P<0.05
TP53 : IM vs LD P<0.05, LD,HD vs AGC P<0.05

Fig. 6. Schematic drawing of the expression profiles of TGFB signaling components and TP53 during the chronic atrophic gastritis-dysplasia-carcinoma sequence. Abbreviations: CA, carcinoma; AD, Adenoma; HD, High grade dysplasia; LD, low grade dysplasia; IM, intestinal metaplasia; CAG, Chronic atrophic gastritis; NL, normal gastric epithelium.

Table 5. Expression patterns of TGFB signaling molecules and TP53 in gastric carcinoma (GC) that have been reported in the literature.

	Expression	genetic/epigenetic alterations	Our data#
TGFB1/2	23-79% positive in GC (Maehara et al., 1999; Saito et al., 1999; Ebert et al., 2000; Xiangming et al., 2001; Zolota et al., 2002)	—	30.0% strong positive in GC
TGFBR1	82% downregulation in GC (Ito et al., 1992)	13-64% methylation (Kang et al., 1999; Pinto et al., 2003)	49.5% strong positive in GC
TGFBR2	42% downregulation in GC (Takeno et al., 2002)	33%LOH 0-74% mutation (Guo et al., 1998; Takeno et al., 2002)	21.1% strong positive in GC
SMAD2/3	38% downregulation in GC (SMAD3) (Han et al., 2004)	no mutation (SMAD2) (Shitara et al., 1999)	42.2% downregulation in GC*
SMAD4	13-75% downregulation in GC (Xiangming et al., 2001; Kim et al., 2004)	10% mutation, 29-45% LOH, 5% methylation (Wang et al., 2007)	50.9% downregulation in GC*
SMAD7	32% positive in GC (Kim et al., 2004)		37.3% strong positive in GC
CDKN1A	30-62% downregulation in GC (Jang et al., 1998; Park et al., 1998; Xie et al., 2004; Al-Moundhri et al., 2005)	no mutation Park et al., 1998 27% polymorphism (Xie et al., 2004)	20.2% downregulation in GC*
CDKN1B	52-60% downregulation in GC (Jang et al., 1998; Al-Moundhri et al., 2005)	no mutation (Shin et al., 2000)	60.0% downregulation in GC*
MYC	47-69% overexpression in GC (Spandidos et al., 1991; Onoda et al., 1996; Hara et al., 1998; Han et al., 1999; Kozma et al., 2001)	Amplification (Hara et al., 1998; Kozma et al., 2001)	40.5% strong positive in GC
CDC25A	38% overexpression in GC (Kudo et al., 1997)		40.7% strong positive in GC
RELA	70% positive in GC (Sasaki et al., 2001)		54.5% strong positive in GC
TP53	50% overexpression in GC (Fenoglio-Preiser et al., 2003)	37%LOH 34%mutation (Fenoglio-Preiser et al., 2003)	26.1% strong positive in GC

obtained from Supplementary Table 2, * Nuclear staining.

CDC25A, and RELA up-regulation was significantly correlated with increased tumor size, whereas SMAD1/2/3(N) and SMAD4(N) were inversely correlated with tumor size (Table 4).

Discussion

In this study, we have demonstrated dynamic changes in expression of TGFB signaling components and TP53 in gastric adenocarcinoma and related lesions. Based on our findings, our hypothesis regarding the change in character of TGFB signaling during the tumorigenesis of gastric neoplasms is illustrated in Fig. 5.

CAG, metaplasia, and dysplasia

In CAG, we noted increased expression of both pro-oncogenic (SMAD7, RELA) and tumor suppressor proteins, including SMAD1/2/3(C), SMAD2/3(C) SMAD4(C/N), CDKN1A, and CDKN1B (Fig. 6). In dysplasia, more extensive changes in the expression profile were observed, and they are also characterized by elevated expression of both pro-oncogenic (TGFB, TGFBR1, TGFBR2) and tumor suppressor proteins, including SMAD7, RELA, SMAD1/2/3, SMAD4, CDKN1A, and CDKN1B. In CAG and dysplasia, TGFB1 signaling possibly modulates the direct induction of CDKN1A gene transcription (Pardali et al., 2000) and the regulation of CDKN1B (Sandhu et al., 1997) via a SMAD-dependent pathway. In addition, SMAD7 up-regulation in CAG and dysplasia could be related to the induction of transient expression of SMAD7 by negative feedback modulation of a TGFB1 signal (Li et al., 2005). In CAG and dysplasia, epithelia may try to counteract various cell proliferation stimuli by activation of TGFB1 signaling. Moreover, the enhanced expression of TGFB and its receptors may activate pro-oncogenic, SMAD-independent pathways, particularly Ras/MAPK/MEK signaling, as well as tumor suppressive, SMAD-dependent pathways (Wakefield and Roberts, 2002) (Fig. 5). Therefore, the overall effects of TGFB signaling in CAG and dysplasia may be unaltered due to the balance between pro-oncogenic and tumor suppressor activities (Fig. 5).

Comparison of our results with previous studies of the expression of TGFB signaling proteins in GC

Our results are generally well consistent with previous studies. The high expression of TGFB in AGC has been reported in previous studies (Kai et al., 1996;

Naef et al., 1997) and has been positively correlated with invasion and metastasis, in accordance with previous studies (Maehara et al., 1999; Saito et al., 1999) (Table 5). There are very few reports which have examined TGFBR expression in GC tissues. According to Tateishi et al., TGFBR1 and TGFBR2 were expressed in 32% and 18% of GC tissues, respectively, and TGFBR2 expression was correlated with invasion and poor prognosis (Tateishi et al., 2000). Several studies on human GC cell lines have revealed that loss of TGFBR1 expression and decreased expression of TGFBR2, or expression of a truncated form of TGFBR2, may play an important role in the inhibition of apoptosis (Yang et al., 1999; Zhuang et al., 1999; Park et al., 2001) and that homozygous mutation of TGFBR2 is observed in two of nine GC cell lines (unpublished data), although down-regulation of TGFBR1 and TGFBR2 in GC patients has not been investigated in detail. A homozygous mutation of TGFBR2, targeting a polyadenine tract in its coding sequence, could represent a replication-error (RER)-positive carcinoma, contributing to 10% of GC (Guo et al., 1998). Moreover, a paradoxical increase in TGFB signaling has been shown in individuals with various congenital anomalies by heterozygous mutation in TGFBR1 or TGFBR2, and transgenic mice expressing a dominant negative (kinase-domain deleted) TGFBR2 (Denton et al., 2003; Loeys et al., 2005). Recently, a TGFBR1 inhibitor has been reported to prevent the invasion and metastasis of SMAD4-deficient pancreatic carcinoma cells (Subramanian et al., 2004), which further suggests the significance of increased expression of TGFBR1 and TGFBR2 in tumorigenesis.

Decreased expression of SMAD3 in 37.5% of GCs and restoration of TGFB responsiveness by introduction of SMAD3 into tumor cells has been reported by Han et al. (2004). These results are also consistent with the data presented here (Table 5). Currently, we have confirmed that SMAD4 down-regulation is critical for progression of GC (Wang et al., 2007). Our data support the hypothesis that the alteration of TGFB signaling in GC is primarily generated not by TGFR2 inactivation, but by SMADs inactivation via various mechanisms (Wang et al., 2007) (Table 5).

The high level of CDKN1A expression in dysplasia and the subsequent decrease in GC was reported by Xie et al. (Xie et al., 2004; Sun et al., 2005) (Table 5). Reduced expression of CDKN1B was reported in 76% of GCs without correlation with clinico-pathological parameters (Wiksten et al., 2002) (Table 5). Furthermore, Helicobacter pylori infection in CDKN1B knockout mice was associated with significantly higher incidences of IM, dysplasia, and carcinoma in the stomach (Kuzushita et al., 2005). Kudo et al. reported that the expression of CDC25A was found in 38% of gastric carcinomas without clinical significance (Kudo et al., 1997) (Table 5). The frequent expression of MYC and its relevance to advanced stages of carcinoma was reported by Spandidos et al. (Spandidos et al., 1991) (Table 5).

Overexpression of TP53 in GC and its relevance to a poor prognosis has been reported in several papers (Okuyama et al., 2002; Xie et al., 2004; Al-Moundhri et al., 2005; Sun et al., 2005), the results of which are consistent with our data here. The expression of RELA has been reported in GC, although the clinical significance of RELA in GC is controversial. Yamanaka et al. and Sasaki et al. reported that RELA expression was correlated with poor prognosis, whereas the results of Lee et al. indicate the exact opposite, i.e., RELA expression was associated with better prognosis (Sasaki et al., 2001; Yamanaka et al., 2004; Lee et al., 2005). Our results are similar to those of Yamanaka et al. and Sasaki et al. in that RELA expression was correlated with greater tumor size and was present at higher levels in patient clusters with poorer prognoses.

The paradoxical function of TGFB signaling in GC

TGFB has the potential to function as a tumor suppressor (via its effects on proliferation, replication potential, and apoptosis), or as a tumor promoter (via its effects on migration, invasion, angiogenesis, and the immune system). How can this dichotomy of function be resolved? Based on animal models and in vitro studies, Elliott and Blobe (2005) proposed a hypothesis that during early tumorigenesis, TGFB-mediated tumor suppressor activity functions through a SMAD-dependent pathway, but that TGFB promoted tumor progression later through a SMAD-independent pathway. However, no complete clinical study has previously supported this hypothesis. In the present study, our data showing the up-regulation of TGFB, TGFBR1, TGFBR2, CDC25A, MYC, and the reduced expression of SMAD1/2/3(N), SMAD2/3(N), SMAD4(C), SMAD4(N) in AGC (Fig. 6), is consistent with Elliott's hypothesis. Consequently, the prevalence of SMAD-independent pathways due to the down-regulation of SMADs may be strongly pro-oncogenic (Fig. 5). TGFB-dependent down-regulation of MYC is central to its ability to inhibit proliferation in many cells (Chen et al., 2001). In addition, repression of MYC in TGFB signaling is known to result from the binding of a SMAD complex to the MYC promoter (Chen et al., 2001). Therefore, up-regulation of MYC in AGC may be central to the functional disruption of the SMAD-dependent pathway (Fig. 5). At this point, it is noteworthy that nuclear expression of SMAD1/2/3, SMAD2/3, and SMAD4 was reduced only in advanced stages (Fig. 6).

In conclusion, we have comprehensively and systematically examined the expression of TGFB signaling components, emphasizing the SMAD-dependent pathway in GC and its precursor lesions. There were extensive alterations in the expression of TGFB signaling molecules and TP53 across the whole gastritis-dysplasia-carcinoma sequence, and our results suggest that the fundamental change in TGFB pathway signals from tumor suppressive to pro-oncogenic may be

due to down-regulation of SMADs concomitant with tumor progression. These findings may be valuable in understanding the mechanisms of GC.

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