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Expression analysis of Wnt pathway associated genes in gastric cancer as a prognostic factor

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ABSTRACT

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Introduction
Gastric cancer (GC) is heterogeneous disease in terms of its histological findings and biological processes. It has recently been divided into four molecular subgroups, also involved in Wnt/β-catenin signaling pathway. However, the relationship between other Wnt-related proteins and the prognosis of gastric cancer has yet to be fully elucidated.
Methods
A large sample size (1158 cases of advanced GC (AGC)) was investigated using immunohistochemical staining and tissue microarray for the expression of CDX-1, CDX-2, XPNPEP3, TRIM24, glutamine synthetase (GS), BMP-4, AXIN2, and TCF4.
Results
Of the total cases, 69 (5.9%) were found to be EBV-positive, 113 (9.7%) were mismatch repair deficient (MMR-d), and the remaining 978 (84.3%) were EBV-negative MMR proficient (MMR-p) AGCs. In the EBV-positive and MMR-d groups, the expression of CDX-1 and CDX-2 had no relationship with patient outcome. Among the 978 EBV-MSS AGCs, 537 (54.9%) and 418 (42.7%) GCs were positive for CDX-2 and CDX-1 expression. Survival analysis demonstrated that CDX-1 and CDX-2 expression was correlated with a favorable patient outcomes in terms of overall survival (multivariate analysis; p-value = 0.018 and 0.028). However, other Wnt-related proteins, including XPNPEP3, TRIM24, GS, BMP-4, AXIN2, and TCF4, were not. The expression of CDX-2 and CDX-1 was associated with a favorable outcome in EBV-MSS intestinal/mixed and diffuse type GCs, respectively (log-rank p-value: 0.010, 0.042 and 0.017, 0.017).
Conclusions
The expression of CDX-1 and CDX-2 are favorable prognostic factors in EBV-negative MMR-p AGC.

Key words : CDX-1, CDX-2, gastric cancer, Wnt/β-catenin signaling pathway

Expression analysis of Wnt pathway associated genes in gastric cancer as a prognostic factor

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I. INTRODUCTION

Gastric cancer (GC) is heterogeneous disease in terms of the histological findings and biological processes associated with it, especially in the advanced stages of this disease [1]. Several clinical studies have found that the biological processes and prognosis associated with GC can vary significantly between patients in the same stages of disease. According to research by The Cancer Genome Atlas (TCGA), GCs can be divided into four molecular subgroups: (1) tumors positive for Epstein–Barr virus, which display *PIK3CA* mutation, extreme DNA hypermethylation, and amplification of *JAK2*, *PD-L1* and *PD-L2*; (2) microsatellite unstable tumors, which show elevated rates of oncogenic signaling protein mutations; (3) genomically stable (GS) tumors, which show various histologic features and frequent mutations of *CDH1* and *RHOA* mutations; (4) tumors with chromosomal instability (CIN), which show marked aneuploidy and focal amplification of receptor tyrosine kinases [2].

Wnt signaling pathways are known to regulate embryonic development, cell growth, differentiation and carcinogenesis. The canonical pathway is activated by extracellular WNT ligand. In the presence of extracellular WNT ligand, WNT ligands bind to the Frizzled (FZD) receptor, resulting in the disengagement or disruption of the destruction complex, composed of

adenomatous polyposis coli (APC), axin, casein kinase-1 (CK-1), and glycogen synthase kinase-3 (GSK-3), allowing cytosolic β-catenin to accumulate and translocate into the nucleus. β-catenin is associated with the T-cell factor (TCF)/lymphoid enhancer factor (LEF) family of transcription factors and induces gene transcription [3].

Recently, organoid technology has emerged as an option for culturing patient-derived GCs, which allows for the proliferation of human gastric epithelium using stem cell "niche factors," which are essential for the self-renewal of gastric epithelial stem cells, including Wnt-3A and R-spondin (Wnt signal activators), EGF, FGF10, Noggin (a BMP inhibitor), and A83-01 (a TGF-b inhibitor) [4]. In a study published in 2018 using organoids, the investigators suggested that gastric cancers could be divided into 4 phenotypes: Wnt/R-spondin-dependent,R-spondin-independent, Wnt/R-spondin-independent, Wnt signal-independent. They also suggested that the Wnt signaling pathway could be associated with gastric cancerogenesis via genetic or epigenetic gene alterations [5]. Their findings suggest that the levels of Wnt activity could vary according to molecular subtypes of GC. As such, patient outcome would also vary, according to the Wnt activity in the different molecular subtypes. However, there is currently not enough research on the activity of Wnt-related proteins in the molecular subtypes of GC to warrant this conclusion.

The aim of this study was to evaluate expression of Wnt-related proteins by immunohistochemical (IHC) staining to determine the association between Wnt-related protein expression and the clinicopathological features of gastric cancer in patient survival, in the different molecular subtypes of GC.

II. MATERIALS AND METHODS

1. Patients and tissue collection

A total of 1,158 patients with advanced gastric cancer (AGC) (760 males and 398 females) who underwent a gastrectomy with D2 lymph node dissection at

Yonsei University College of Medicine from January 2000 to December 2003 were consecutively enrolled. Patients who had undergone preoperative chemotherapy or radiotherapy and those who had undergone surgery for recurred cancer were excluded. The mean age of the patients was 56.76 years (range, 25-88 years) and the mean follow-up duration was 57.7 months (range, 2.0-109.4 months). Patients' clinical information and survival data were obtained from the medical records and the Korean cancer registry. This study was approved by the Institutional Review Board of Yonsei University College of Medicine (approval number: 4-2014-0668). Tumor histology was classified as differentiated and undifferentiated based on the Japanese gastric cancer treatment guidelines 2010 [26].

2. Tissue microarray construction and immunohistochemical staining

Two cores of tumor tissue (3-mm diameter) were punched out from individual formalin-fixed and paraffin-embedded tumor blocks and arrayed in a new tissue microarray (TMA) block. A core of adjacent non-neoplastic mucosa was arrayed in each TMA block as a landmark and an internal control. The non-neoplastic mucosa core was sampled from the adjacent mucosa in the tumor block. Sections (4-μm thick) from each TMA block were prepared for immunohistochemical (IHC) staining. Hematoxylin and eosin (H&E) and cytokeratin IHC staining were performed to confirm the presence of tumor cells.

Immunohistochemical staining with antibodies caudal-related homeobox transcription factor 1 (CDX-1; 1:100; Abcam,), CDX-2 (1:400; Cell Marque) XPNPEP3 (1:100; Abcam), TRIM24 (1:100; Atlas Antibodies), Glutamine synthetase (GS) (1:100; Roche), Bone morphogenetic protein 4 (BMP-4) (1:100; Merck Group), AXIN2 (1:500; Abcam), and TCF4 (1:100; Atlas Antibodies) was carried out using a Ventana Discovery XT automated staining system (Ventana Medical Systems, Tucson, AZ, USA).

The stained TMA slides were independently reviewed by two pathologists (S.

Noh, K. Kim) who were unaware of any of the patients' medical information. The expression patterns of CDX-1, CDX-2, and TRIM24 were categorized into three groups: (1) strong, in which staining was nuclear and stronger than that of the normal gastric mucosa; (2) equivocal and weak, in which staining was nuclear with a similar or weaker intensity than that of the normal gastric mucosa; (3) absent staining, in which no tumor cells were expressed. The expression patterns of XPNPEP3 were categorized into three groups: (1) strong, in which staining was membranous or cytoplasmic with a stronger intensity than that of the normal gastric mucosa; (2) equivocal and weak, in which staining was membranous or cytoplasmic with a similar or weaker intensity than that of normal gastric mucosa; (3) absent, no tumor cells were expressed. The expression patterns of GS and BMP-4 were categorized into three groups: (1) strong; staining was nuclear or cytoplasmic with a stronger intensity than that of the normal gastric mucosa; (2) equivocal and weak, in which staining was nuclear or cytoplasmic with a similar or weaker intensity than that of the normal gastric mucosa; (3) absent, in which no tumor cells were expressed. The expression patterns of AXIN2 and TCF4 were categorized into three groups: (1) strong and diffuse, in which staining was nuclear or cytoplasmic with a stronger intensity than that of normal gastric mucosa, or a positive staining of over 50% of all the area of tumor cells; (2) equivocal or focal, in which staining was nuclear or cytoplasmic with a similar intensity to that of the normal gastric mucosa, or a positive staining of under 50% of all the area of tumor cells; (3) absent, in which no tumor cells were expressed.

3. WNT-related proteins

We selected markers related to the WNT/β-catenin pathway. In Nusse's database (https://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes), we searched for Wnt-related genes. In the protein database (https://www.proteinatlas.org), we investigated whether antibodies for the gene were present, immunohistochemical staining was available, and

immunohistochemical staining expression varied. The following antibodies were selected for the Wnt-related proteins:

A. CDX-1 and CDX-2

CDX-1 and CDX-2 (CDXs), members of the caudal-related homeobox gene family, are intestine-specific transcriptional factors related to the proliferation and differentiation of intestine epithelial cells. Several reports have found that CDX-2 inhibits the proliferation and carcinogenesis of colon cancer by suppressing β-catenin signaling [6,7]. One study suggested that CDX-2 attenuates Wnt/β-catenin signaling by directly transactivating GSK-3β and Axin2 expression in colon cancer cells [6]. As such, CDXs are considered as indirect Wnt signal inhibitors.

B. XPNPEP3

A study identified XPNPEP3 as a novel target of canonical Wnt/β-catenin signaling, making it highly relevant for colorectal cancer through the microarray-based mRNA profiling of rectal cancer samples. Moreover, XPNPEP3 expression and β-catenin nuclear localization have been previously found to exhibit a significant positive correlation in colorectal tumors [8].

C. TRIM24

The tripartite motif (TRIM) family, identified as a subfamily of the RING-type E3 ubiquitin ligase family, is involved in cell growth, apoptosis, cell development, and tumorigenesis [9]. TRIM24 knockdown in human HepG2 liver cancer cells downregulates β-catenin and cyclin D1, two major downstream genes of the Wnt pathway [10]. Therefore, TRIM24 is considered as an indirect Wnt promotor in this study.

D. Glutamine synthetase (GS)

One study found that the overexpression of glutamine synthetase is associated with the activation of β-catenin signaling in the liver [11]. In this study, GS was regarded as a direct Wnt target protein.

E. Bone morphogenetic protein 4 (BMP4)

The expression of BMP-4, a member of the transforming growth factor-β family, was measured in human colon cancer cell lines using a BMP-4 ELISA assay. BMP-4 overexpression was found in human colon cancer cells with mutant APC genes [12]. Here, BMP4 is regarded as a direct Wnt target protein.

F. Axin2

Cytosolic β-catenin is recognized by the destruction complex, composed of Axin, APC, CK-1, and GSK-3. The destruction complex phosphorylates β-catenin, thereby targeting the protein for ubiquitination and subsequent proteasomal degradation.

G. TCF4

Activated β-catenin in the nucleus interacts with members of the T-cell factor (TCF)/lymphoid enhancer factor (LEF-1) family. This complex stimulates proliferation, morphogenesis and the epithelial-mesenchymal transition, and drives carcinogenesis [13]. TCF4 is the main binding partner of β-catenin in the colon and mediates the transformation of colon epithelial cells.

4. Statistical analysis

The clinical and pathological data were analyzed using IBM SPSS software version 20.0 (IBM Corp. Armonk, NY, USA). Pearson's Chi-square test was applied for the analysis of the correlation between clinicopathological variables and Wnt-related protein expression patterns. Overall survival was defined as the time interval from surgery until either the date of tumor-related death from any cause or the date of the last follow-up, wherein disease-free survival was defined as the interval from surgery to the date of recurrence or the date of the last follow-up. Survival curves were estimated using the Kaplan-Meier method and log-rank test. Univariate and multivariate analyses to estimate the independent prognostic significances of Wnt-related protein expression were carried out using Cox regression analysis. Statistical significance was defined as $P<0.05$.

Table 1: Antibodies used for immunohistochemical staining

Antibody	Source	Dilution
CDX-1	Abcam, Gyeonggi-do, Seoul, Rep of Korea	1:100
CDX-2	Cell Marque, Rocklin, CA, USA	1:400
XPNPEP3	Abcam, Gyeonggi-do, Seoul, Rep of Korea	1:100
TRIM24	Atlas Antibodies, Stockholm, Sweden	1:100
Glutamine synthetase	Roche, Seoul, Rep of Korea	1:100
BMP-4	Merck group, Seoul, Rep of Korea	1:100
AXIN2	ABCAM, Gyeonggi-do, Seoul, Rep of Korea	1:500
TCF4	Atlas Antibodies, Stockholm, Sweden	1:100

5. Subgrouping of AGCs

All cases were divided into four subgroups using a so-called "histologic-molecular classification" based on findings retrieved from previous large sample-sized studies on gastric cancer using the same cohort based on the Lauren classification, EBER-ISH results, MMR proteins, and p53 IHC results [14]. The four subgroups were defined as follows: (1) EBV-positive: EBER-ISH positive; (2) MMR-deficient: EBER-ISH negative and MMR-deficient; (3) D-pGS (diffuse-putative genome stable): EBER-ISH negative, MMR-proficient, p53 wild-type pattern, and diffuse type by Lauren classification; (4) I-pCIN (intestinal-putative chromosome instability) (p53 mutant-pattern): EBER-ISH negative, MMR-proficient, and p53 mutant-pattern regardless of histologic type by Lauren classification and I-pCIN (p53 wild-pattern): EBER-ISH negative, MMR-proficient, p53 wild-pattern, and intestinal or mixed type by Lauren classification.

III. RESULTS

1. Expression pattern of Wnt-related proteins

Clinicopathologic characteristics according CDX-1, CDX-2, AXIN2, TCF4, XPNPEP3, TRIM24, BMP-4, and GS IHC results are summarized in Tables 2-3.

Among the total cases, 69 (5.9%) were EBV-positive, 113 (9.7%) were mismatch repair deficient (MMR-d), 159 (13.7%) were D-pGS, and the remaining 819 (70.6%) were I-pCIN-type GCs. Among the 1160 GCs, CDX-1 showed strong expression (2+) in 169 cases, equivocal or weak expression (1+) in 303 cases, and negative expression in 467 cases. CDX-1 was expressed in 19 cases (30.2%) in EBV-positive, 33 cases (32.3%) in MMR-d, 86 cases (60.2%) in D-pGS, and 334 cases (52.9%) in I-pCIN AGCs. CDX-2 showed strong expression (2+) in 249 cases, equivocal or weak expression (1+) in 343 cases, and negative expression in 436 cases. CDX-2 was expressed in 10 cases (14.5%) in EBV-positive, 45 cases (40.5%) in MMR-d, 92 cases (59.4%) in D-pGS, and 445 cases (57.6%) in I-pCIN AGCs. XPNPEP3 showed strong expression (2+) in 255 cases, equivocal or weak expression (1+) in 434 cases, and negative expression in 223 cases. XPNPEP3 was expressed in 54 cases (88.5%) in EBV-positive, 69 cases (31.0%) in MMR-d, 106 cases (76.3%) in D-pGS, and 460 cases (75.2%) in I-pCIN AGCs. TRIM24 showed strong expression (2+) in 148 cases, equivocal or weak expression (1+) in 526 cases, and negative expression in 242 cases. TRIM24 was expressed in 54 cases (88.5%) in EBV-positive, 58 cases (58.0%) in MMR-d, 113 cases (81.3%) in D-pGS, and 449 cases (72.9%) in I-pCIN AGCs. GS showed strong or equivocal expression (2+) in 382 cases, weak expression (1+) in 435 cases, and negative expression in 99 cases. GS was expressed in 56 cases (91.8%) in EBV-positive, 81 cases (81.0%) in MMR-d, 126 cases (90.6%) in D-pGS, and 554 cases (89.9%) in I-pCIN AGCs. BMP-4 showed strong expression (2+) in 216 cases, equivocal or weak expression (1+) in 347 cases, and negative expression in 376 cases. BMP-4 was expressed in 42 cases (67.7%) in EBV-positive, 41 cases (41.0%) in MMR-d, 103 cases (71.5%) in D-pGS, and 377 cases (59.6%) in I-pCIN AGCs. AXIN2 showed strong expression (2+) in 517 (57.4%), focal equivocal expression in 191 (21.2%) cases, and negative expression in 192 (21.3%) cases. AXIN2 was highly expressed (2+) in 21 cases

(46.5%) in EBV-positive, 52 cases (54.2%) in MMR-d, 87 cases (61.7%) in D-pGS, and 351 cases (58.1%) in I-pCIN AGCs. TCF4 showed strong expression (2+) in 134 (14.9%) cases, focal equivocal expression in 309 (34.4%) cases, and negative expression in 456 (50.7%) cases. AXIN2 was highly expressed (2+) in 14 cases (24.6%) in EBV-positive, 15 cases (15.6%) in MMR-d, 16 cases (11.3%) in D-pGS, and 89 cases (14.7%) in I-pCIN AGCs. The immunohistochemical staining results showed significant differences according to the histologic-molecular classification ($p<0.05$) in all cases except for Axin2 ($p = 0.405$).

2. Survival analysis

A. Kaplan-Meier method

The Kaplan-Meier survival curves showed no significant differences for disease-free survival (DFS) and overall survival (OS) in the EBV-positive GCs in terms of Wnt-related protein expression. In MMR-d GCs, the expression of TRIM24, TCF4, and BMP-4 was correlated with a significantly higher OS (p-value = 0.049, 0.047, 0.013, respectively), whereas Axin2 expression was correlated with a significantly favorable DFS (p-value = 0.008). In D-pGS and I-pCIN GCs, the expression of CDX-1 and CDX-2 was correlated with a significantly favorable OS (p-value = 0.024, 0.018 for CDX-1; 0.044, 0.005 for CDX-2). The expression of CDX-2 was correlated with a better DFS in D-pGS and I-pCIN GC (p-value = 0.022, 0.02, respectively); however, CDX-1 expression was correlated with a better DFS only in D-pGS GC (p-value = 0.009). XPNPEP expression was significantly correlated with favorable OS in D-pGS GC (p-value = 0.044), while TRIM24 expression was correlated with favorable DFS in D-pGCS GC (p-value = 0.039). Only CDX-1 and CDX-2 were correlated with both OS and DFS in patients with GC.

Table 2: Clinicopathologic characteristics of advanced gastric cancers according to the expression status of CDX-1, CDX-2, AXIN2, TCF4

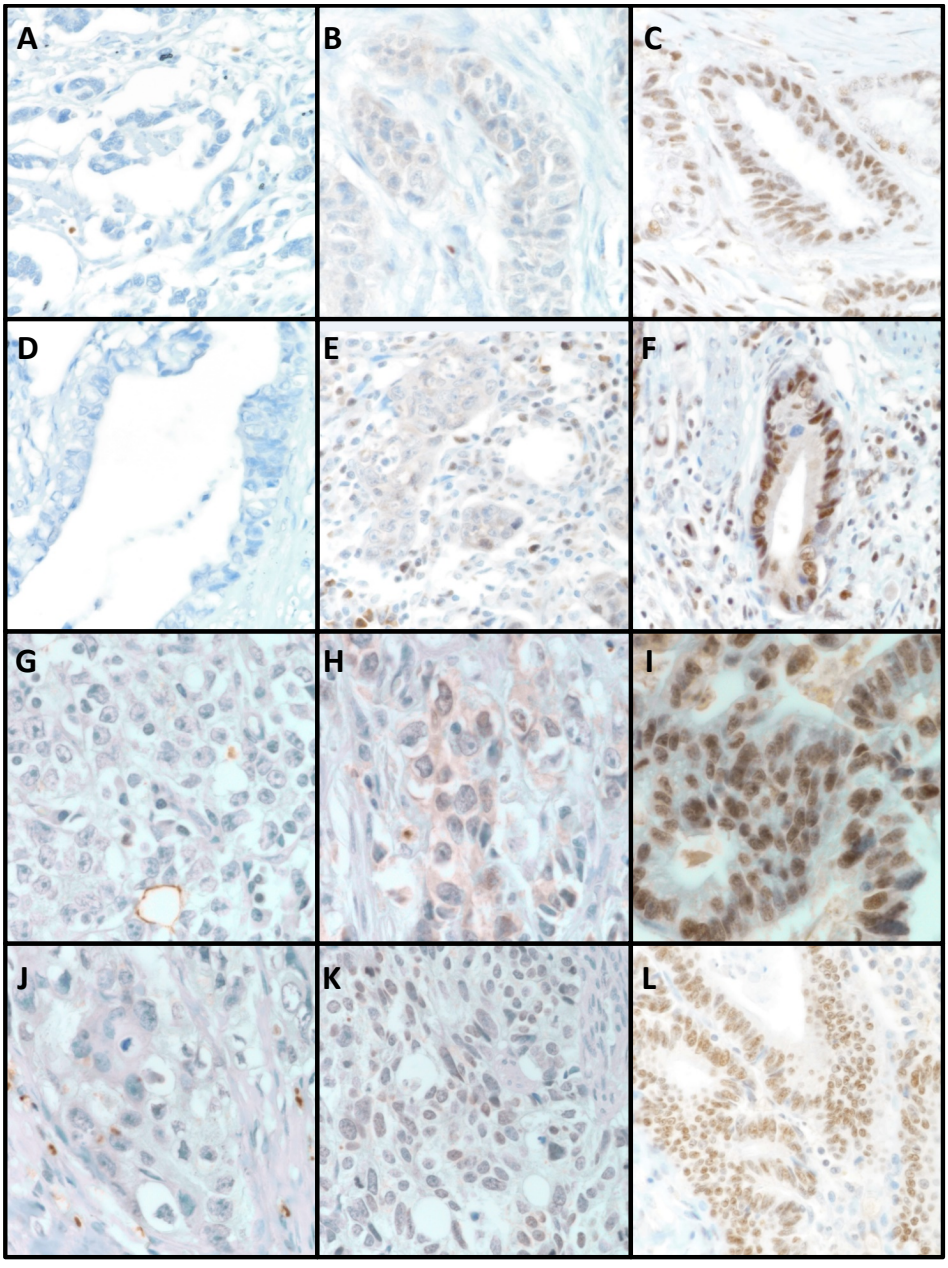
Category	Variables	No. of cases	CDX-1			No. of cases	CDX-2			No. of cases	AXIN2			No. of cases	TCF4		
		(%)	Positive(%)	Negative(%)	P-value		Positive(%)	Negative(%)	P-value		High expression(%)	Low expression(%)	P-value		High expression(%)	Low expression(%)	P-value
Age (years)			57.3±12.3	57.1±11.8	0.779		56.63±12.6	56.94±11.9	0.692		57.2±12.3	57.2±11.9	0.959		57.8±11.5	57.1±12.1	0.495
Sex	Male	618	301 (63.9)	317 (67.9)	0.215	672	386 (65.3)	286(65.6)	0.947	594	344 (66.7)	250 (65.3)	0.67	593	93 (69.9)	500 (65.4)	0.323
	Female	320	170 (36.1)	150 (32.1)		355	205 (34.7)	150 (34.4)		305	172 (33.3)	133 (34.7)		305	40 (34.6)	265 (34.6)	
Differentiation	Differentiated	277	126 (26.8)	151 (32.3)	0.063	293	185 (31.3)	108 (24.8)	0.025	266	154 (29.8)	112 (29.2)	0.883	266	51 (38.1)	215 (28.1)	0.024
	Undifferentiated	661	345 (73.2)	316 (67.7)		735	407 (68.8)	328 (75.2)		633	362 (70.2)	271 (70.8)		632	82 (61.9)	549 (71.9)	
Lauren classification	Intestinal or mixed	501	214 (45.8)	287 (61.7)	<0.001	538	309 (52.4)	229 (52.6)	0.95	472	268 (52.3)	204 (53.4)	0.787	471	86 (65.2)	385 (50.6)	0.002
	Diffuse	431	253 (54.2)	178 (38.3)		488	282 (47.7)	206 (47.4)		422	244 (47.7)	178 (46.6)		422	46 (34.8)	376 (49.4)	
LVI	Absent	664	343 (72.8)	321 (68.7)	0.173	733	430 (72.8)	303 (69.5)	0.264	632	349 (67.6)	283 (73.9)	0.046	633	89 (66.9)	544 (71.1)	0.354
	Present	274	128 (27.2)	146 (31.3)		294	161 (27.2)	133 (30.5)		267	167 (32.4)	100 (26.1)		265	44 (33.1)	221 (28.9)	
LNM	Absent	258	138 (29.2)	120 (25.7)	0.242	286	174 (29.4)	112 (25.7)	0.205	248	136 (26.3)	112 (29.2)	0.365	249	36 (26.9)	213 (27.8)	0.917
	Present	681	334 (70.8)	347 (74.3)		742	418 (70.6)	324 (74.3)		652	381 (73.7)	271 (70.8)		650	98 (73.1)	552 (72.2)	
Pathologic T stage	T2	147	75 (15.9)	72 (15.5)	0.688	169	110 (18.7)	59 (13.5)	0.148	139	74 (14.3)	65 (17)	0.394	140	20 (15.0)	120 (15.7)	0.84
	T3	344	172 (84.1)	172 (84.5)		369	205 (34.8)	164 (37.6)		334	189 (36.6)	145 (38)		333	47 (35.3)	286 (37.4)	
	T4	446	224 (47.6)	222 (47.6)		487	274 (46.5)	213 (48.9)		425	253 (49.0)	172 (45)		424	66 (49.6)	358 (46.9)	
p53 IHC	Wild-type pattern	345	178 (39.1)	167 (36.4)	0.285	380	232 (40.1)	148 (34.4)	0.035	336	205 (40.9)	131 (34.7)	0.068	336	50 (38.2)	286 (38.3)	1.000
	Mutant pattern	569	277 (70.1)	292 (63.6)		628	346 (59.9)	282 (65.6)		542	296 (59.1)	246 (65.3)		541	81 (61.8)	460 (61.7)	
EBER-ISH	Negative	853	439 (95.9)	414 (95.4)	0.002	947	579 (98.3)	368 (86.2)	<0.001	822	480 (94.7)	342 (91.7)	0.098	822	118 (89.4)	704 (94.2)	0.053
	Positive	63	19 (4.1)	44 (4.6)		69	10 (1.7)	59 (13.8)		58	27 (5.3)	31 (8.3)		57	14 (10.6)	43 (5.8)	
MMR protein IHC	MMR-proficient	814	425 (92.8)	389 (84.9)	<0.001	905	544 (92.4)	361 (84.5)	<0.001	784	455 (89.7)	329 (88.1)	0.512	783	117 (88.6)	666 (89.2)	0.88
	MMR-deficient	102	33 (7.2)	69 (15.1)		111	45 (7.6)	66 (15.5)		96	52 (10.3)	44 (11.9)		96	15 (11.4)	81 (10.8)	

LVI: lymphovascular invasion, LNM: lymph node metastasis, IHC: immunohistochemistry, EBER-ISH: Epstein-Barr virus encoding RNA in situ hybridization, MMR protein: mismatch repair gene related protein

Table 3: Clinicopathologic characteristics of advanced gastric cancers according to the expression status of XPNPEP3, TRIM24, BMP-4, GS

Category	Variables	No. of cases	XPNPEP3			No. of cases	TRIM24			No. of cases	BMP-4			No. of cases	GS		
			Positive(%)	Negative(%)	P-value		Positive(%)	Negative(%)	P-value		Positive(%)	Negative(%)	P-value		Positive(%)	Negative(%)	P-value
Age (years)			57.3±12.0	57.1±12.3	0.794		57.1±12.1	57.7±11.8	0.502		56.6±11.9	58.1±12.2	0.066		57.4±11.9	56.1±13.4	0.345
Sex	Male	600	458 (66.6)	142 (63.7)	0.465	600	440 (65.4)	160 (66.4)	0.813	618	370 (65.8)	248 (66.0)	1.000	602	538 (65.9)	64 (64.6)	0.823
	Female	311	230 (33.4)	81 (36.3)		314	233 (34.6)	81 (33.6)		320	192 (34.2)	128 (34.0)		313	278 (34.1)	35 (35.4)	
Differentiation	Differentiated	271	201 (29.2)	70 (31.5)	0.501	272	187 (27.7)	85 (35.4)	0.027	275	149 (26.5)	126 (33.5)	0.023	272	244 (29.9)	28 (28.6)	0.907
	Undifferentiated	640	488 (70.8)	152 (68.5)		642	487 (72.3)	155 (64.6)		663	413 (73.5)	250 (66.5)		643	573 (70.1)	70 (71.4)	
Lauren classification	Intestinal or mixed	488	370 (54.0)	118 (53.6)	0.938	490	343 (51.3)	147 (61.3)	0.008	496	282 (50.5)	214 (57.2)	0.052	489	441 (54.2)	48 (50)	0.45
	Diffuse	417	315 (46.0)	102 (46.4)		418	325 (48.7)	93 (38.7)		436	276 (49.5)	160 (42.8)		420	372 (45.8)	48 (50)	
LVI	Absent	641	481 (69.9)	160 (71.7)	0.673	644	464 (68.9)	180 (74.7)	0.1	665	399 (71.0)	266 (70.7)	0.942	645	582 (71.3)	63 (63.6)	0.129
	Present	270	207 (30.1)	63 (28.3)		270	209 (31.1)	61 (25.3)		273	163 (29.0)	110 (29.3)		270	234 (28.7)	36 (26.4)	
LNM	Absent	249	177 (25.7)	72 (32.3)	0.058	253	192 (28.5)	61 (25.3)	0.357	260	157 (27.9)	103 (27.4)	0.882	252	226 (27.7)	26 (26.3)	0.813
	Present	663	512 (74.3)	151 (67.7)		662	482 (71.5)	180 (74.7)		679	406 (72.1)	273 (72.6)		664	591 (72.3)	73 (73.7)	
Pathologic T stage	T2	141	115 (16.7)	26 (11.7)	0.098	145	123 (18.3)	22 (9.2)	0.004	148	85 (15.1)	63 (16.8)	0.615	143	132 (16.2)	11 (11.1)	0.413
	T3	333	241 (35.1)	92 (41.4)		333	237 (35.2)	96 (40.2)		346	214 (38.1)	132 (35.2)		335	296 (36.4)	39 (39.4)	
	T4	435	331 (48.2)	104 (46.8)		434	313 (46.5)	121 (50.6)		443	263 (46.8)	180 (48.0)		435	386 (47.4)	49 (49.5)	
p53 IHC	Wild-type pattern	340	263 (39.1)	77 (35.3)	0.336	338	257 (39.1)	81 (34.6)	0.24	344	214 (39.3)	130 (35.3)	0.237	340	311 (39.1)	29 (29.9)	0.096
	Mutant pattern	550	409 (60.9)	141 (64.7)		553	400 (60.9)	153 (65.4)		569	331 (60.7)	238 (64.7)		553	485 (60.9)	68 (70.1)	
EBER-ISH	Negative	830	620 (92.0)	210 (96.8)	0.013	831	609 (92.1)	222 (96.1)	0.048	854	507 (92.3)	347 (94.6)	0.227	833	743 (93.0)	90 (94.7)	0.669
	Positive	61	54 (8.0)	7 (3.2)		61	52 (7.9)	9 (3.9)		62	42 (7.7)	20 (5.4)		61	56 (7.)	5 (5.3)	
MMR protein IHC	MMR-proficient	791	605 (89.8)	186 (89.7)	0.108	792	602 (91.1)	190 (82.3)	0.001	816	508 (92.5)	308 (83.9)	<0.001	794	718 (89.9)	76 (80.0)	0.009
	MMR-deficient	100	69 (10.2)	31 (10.3)		100	59 (8.9)	41 (17.7)		100	41 (7.5)	59 (16.1)		100	81 (10.1)	19 (20.0)	

LVI: lymphovascular invasion, LNM: lymph node metastasis, IHC: immunohistochemistry, EBER-ISH: Epstein-Barr virus encoding RNA in situ hybridization, MMR protein: mismatch repair gene related protein



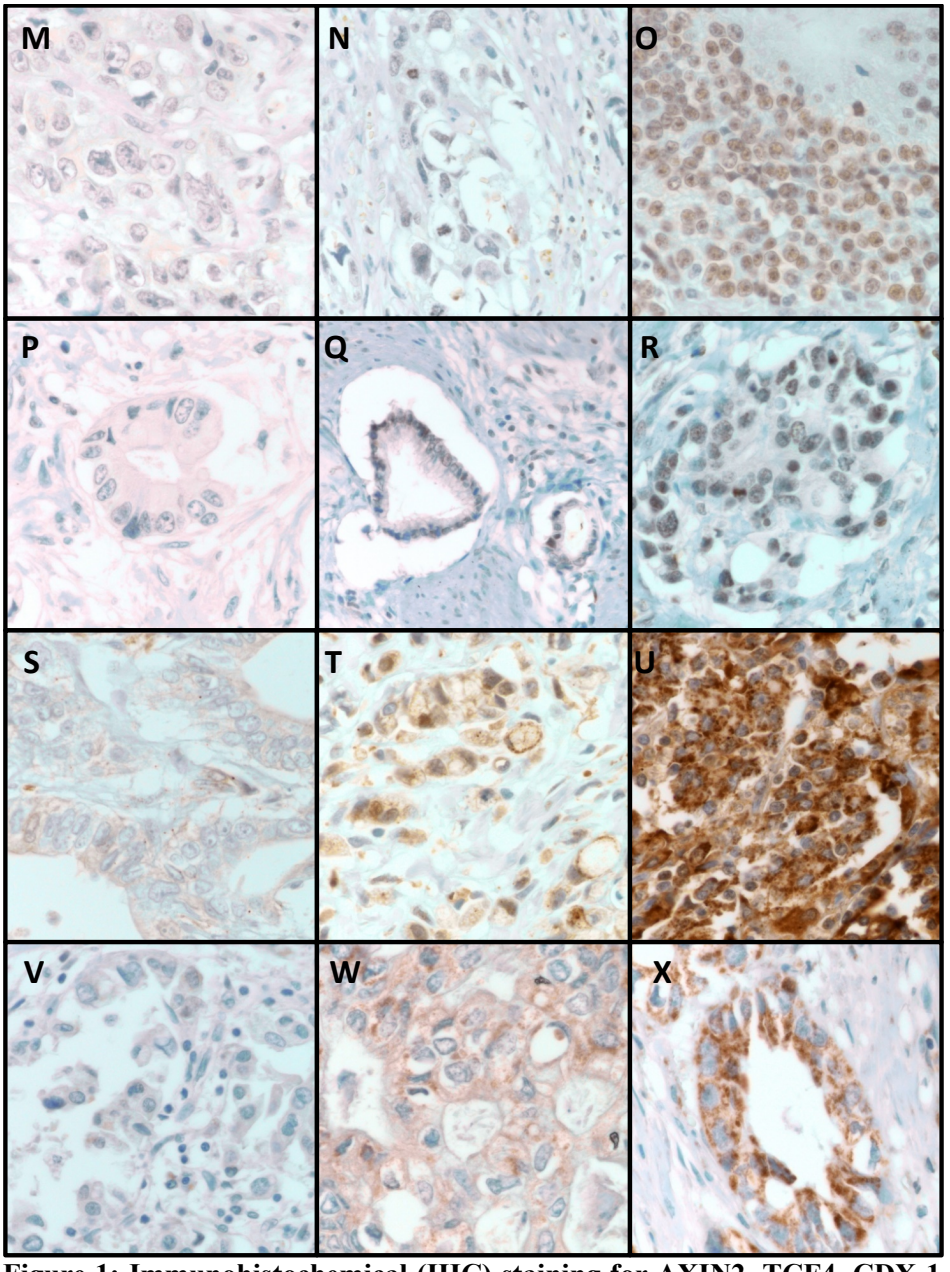


Figure 1: Immunohistochemical (IHC) staining for AXIN2, TCF4, CDX-1, CDX-2, TRIM24, BMP4, GS and XPNPEP3

Representative microphotographs of negative, 1+, and 2+ cases for AXIN2, TCF4, CDX-1, CDX-2, TRIM24, BMP4, GS, and XPNPEP3 (A-C, AXIN2; D-F, TCF4; G-I, CDX-1; J-L, CDX-2; M-O, TRIM24; P-R, BMP4; S-U, GS; V-X, XPNPEP3) with original magnification of 400×.

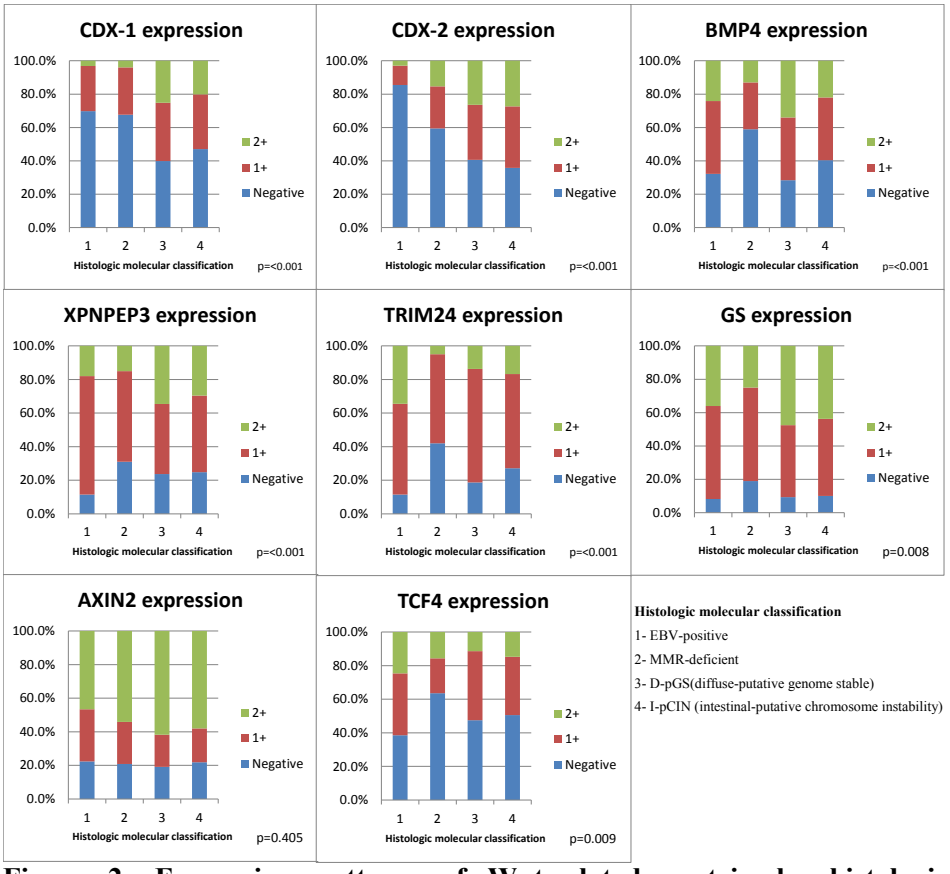


Figure 2: Expression patterns of Wnt-related protein by histologic molecular classification

B. Univariate and multivariate analysis

In EBV-positive and MMR-d GCs, univariate and multivariate analysis showed no significant differences in terms of DFS and OS. In EBV-negative MMR-p AGCs, both CDX-1 and CDX-2 expression were correlated with favorable OS in univariate analysis (p-value = 0.003, 0.001). Moreover, CDX-1, CDX-2, TRIM24, and GS expression were correlated with favorable DFS in univariate analysis (p-value = 0.011, 0.002, 0.024, 0.049). In multivariate analysis, CDX-1 and CDX-2 expression showed a better prognosis in terms of OS (p-value = 0.018, 0.028), while CDX-1 and GS showed in a better prognosis

for DFS (p-value = 0.019, 0.45). The results of other Wnt-related protein expression results are summarized in Table 4.

C. Relationship between CDXs and Lauren classification

The expression of CDX-1 and CDX-2 was associated with a favorable OS in EBV- MSS intestinal/mixed and diffuse type GCs (p-value= 0.017, 0.017 and 0.010, 0.042, respectively). In DFS, the expression of CDX-1 and CDX-2 was significantly associated with a favorable outcome in I-pCIN GCs (p-value = 0.001, 0.037)

IV. DISCUSSION

CDXs are involved in the development and progression of gastric cancer [15,16]. Some studies demonstrated the role of CDX-1 and CDX-2 in the development of intestinal metaplasia in the stomach using a transgenic mouse model [17,18]. Whether CDX-2-positive expression could be considered as a prognostic factor in patients with gastric cancer remains a controversial issue. Several reports have suggested that CDX-2 expression is correlated with good prognostic features in gastric cancer. For example, some studies have demonstrated that the expression of CDX-2 in gastric cancer was associated with a better differentiation and lower rate of lymph node metastasis [19,20]. Other studies have found that CDX-2 expression reduced cell proliferation rates, and that CDX-2-positive expression was decreased progressively with the depth of tumor invasion and the progress of the advanced stages of gastric cancer [21,22]. However, another study suggested that there is no significant correlation between CDX-2 and clinicopathological parameters [23]. Recently, a meta-analysis study indicated that CDX-2 overexpression was significantly associated with a lower clinical stage and a higher 5-year survival rate [15]. However, the sample sizes of the meta-analysis were small. Our large-sized study demonstrated that the CDX-positive group was correlated with a superior patient survival than the CDX-negative group in EBV-negative MMR-p GCs.

Furthermore, a prognostic factor of CDX-positivity was also applied in diffuse type GC, in addition to intestinal/mixed type GC. These findings were similar to studies that suggested the prognostic effect of CDX-2 and CDX-1. However, the molecular networks relating CDX-2 to its function and regulation in gastric cancer remain largely unknown. There is currently an insufficient amount of research being carried out on the role of CDX-2 and CDX-1 in gastric cancerogenesis.

Table 4: Univariate and multivariate survival analysis of EBV-negative MMR-proficient advanced gastric cancers

Factors		Overall survival						Disease free survival					
		Univariate			Multivariate			Univariate			Multivariate		
		HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value
Age (yr)				<0.001			<0.001			0.656			
	≤55	1			1			1					
	>55	1.374	1.156-1.633		1.53	1.247-1.886		0.925	0.762-1.123				
Sex				0.099						0.036			0.604
	Male	1						1			1		
	Female	1.156	0.973-1.374					1.276	1.047-1.555		1.066	0.838-1.355	
Differentiation				<0.001			0.22			<0.001			0.17
	Differentiated	1			1			1			1		
	Undifferentiated	1.536	1.262-1.869		1.198	0.896-1.602		1.759	1.387-2.231		1.29	0.897-1.855	
Lauren classification				0.008			0.622			<0.001			0.297
	Intestinal or mixed	1			1			1			1		
	Diffuse	1.26	1.063-1.493		1.068	0.823-1.385		1.539	1.260-1.880		1.182	0.864-1.617	
Pathologic T stage				<0.001			<0.001			<0.001			<0.001
	2/3	1			1			1			1		
	4	2.549	2.142-3.035		2.173	1.772-2.665		3.124	2.541-3.841		2.355	1.844-3.008	
LVI				<0.001			0.003			<0.001			0.007
	Absent	1			1			1			1		
	Present	2.167	1.818-2.584		1.378	1.117-1.701		2.21	1.805-2.706		1.398	1.095-1.785	
Lymph node metastasis				<0.001			<0.001			<0.001			0.001
	Absent	1			1			1			1		
	Present	3.243	2.591-4.061		2.414	1.824-3.194		3.8	2.887-5.001		2.567	1.837-3.586	
CDX-1				0.003			0.018			0.011			0.019
	Negative	1			1			1			1		
	Expressed	0.756	0.629-0.909		0.778	0.632-0.958		0.759	0.614-0.938		0.749	0.588-0.954	
CDX-2				0.001			0.028			0.002			0.255
	Negative	1			1			1			1		
	Expressed	0.733	0.614-0.876		0.792	0.643-0.975		0.722	0.588-0.885		0.868	0.680-1.107	
XPNPEP3				0.24						0.526			
	Negative	1						1					
	Expressed	0.881	0.713-1.088					0.924	0.723-1.180				
TRIM24				0.099						0.024			
	Negative	1						1					
	Expressed	0.839	0.681-1.034					0.761	0.601-0.964				
GS				0.087						0.049			0.45
	Negative	1						1			1		
	Expressed	0.775	0.579-1.038					0.721	0.520-0.999		0.874	0.617-1.239	
BMP_4				0.176						0.382			
	Negative	1						1					
	Expressed	0.879	0.729-1.060					0.909	0.733-1.126				
AXIN2				0.211						0.573			
	Low expression	1						1					
	High expression	1.13	0.933-1.368					1.065	0.856-1.324				
TCF4				0.475						0.613			
	Low expression	1						1					
	High expression	1.103	0.843-1.443					1.084	0.794-1.479				

† HR, hazard ratio; ‡ 95% CI, 95% confidence interval; LVI, lymphovascular invasion

In another study, XPNPEP3 expression and β-catenin nuclear localization

exhibited a significant positive correlation with colorectal tumors [8], suggesting that a higher XPNPEP3 expression in gastric cancer could be related with overactivated WNT/beta-catenin pathway and a poor prognosis. However, our study demonstrated that XPNPEP3-positive group was correlated with a better patient survival than the XPNPEP3-negative group, which is in contrast to existing findings. The other Wnt-related proteins, that is GS, BMP-4, and TRIM24, did not show any significant correlation with patient outcome. However, there was tendency for groups expressing these proteins to be correlated with a better patient survival than the non-expression groups. This is also in contrast to findings reported by previous studies [24,25]. One study investigating TRIM24 expression found a correlation between the expression of TRIM24 and β-catenin in GC tissues, wherein a high nuclear expression of TRIM24 was correlated with worse rates of survival in patients with GC [24]. However, in our study, the TRIM24-positive group was found to be correlated with a better patient survival compared to the TRIM24-negative group, which is, once again, in contrast to existing findings.

In other study, XPNPEP3 expression and β-catenin nuclear localization exhibited significant positive correlation in colorectal tumor [8]. So it was predicted that higher XPNPEP3 expression in gastric cancer maybe related with overactivated WNT/beta-catenin pathway, and poor prognosis. But our study demonstrated that XPNPEP3-positive group had correlation with better patients' survival rather than XPNPEP3-negative group. It is against finding to existing findings. Other Wnt related proteins, GS, BMP-4, TRIM24 did not have significant relationship with patients' outcome. But they showed a tendency that expression of protein showed better patients' survival rather than non-expression group. It is against finding of other studies [24 25]. A study for TRIM24 expression demonstrated that a correlation was found between the expression of TRIM24 and β-catenin in GC tissues, and high nuclear expression of TRIM24 was correlated with worse survival rates in patients with

GC [24]. But our study showed TRIM24-positive group had correlation with better patients' survival rather than TRIM24-negative group. It is against finding to existing findings.

Our cohort excluded patients who had previously undergone pre-operative chemo- or radiotherapy, as well as patients who had undergone surgery for a recurred cancer. However, patients who had received adjuvant chemotherapy were not excluded. Although CDX-1 and CDX-2 expression showed was statistically significantly found to be prognostic factor, post-operative therapeutic effects could be a confounding factor in patients' survival.

V. CONCLUSION

In conclusion, the expression of CDX-1 or CDX-2 are favorable prognostic factors in EBV-negative MMR-p AGC. We were not able to identify the relationship between the expression intensity of other Wnt-related protein and prognosis of stomach cancer. Further studies will be required to elucidate the underlying mechanism of this result.

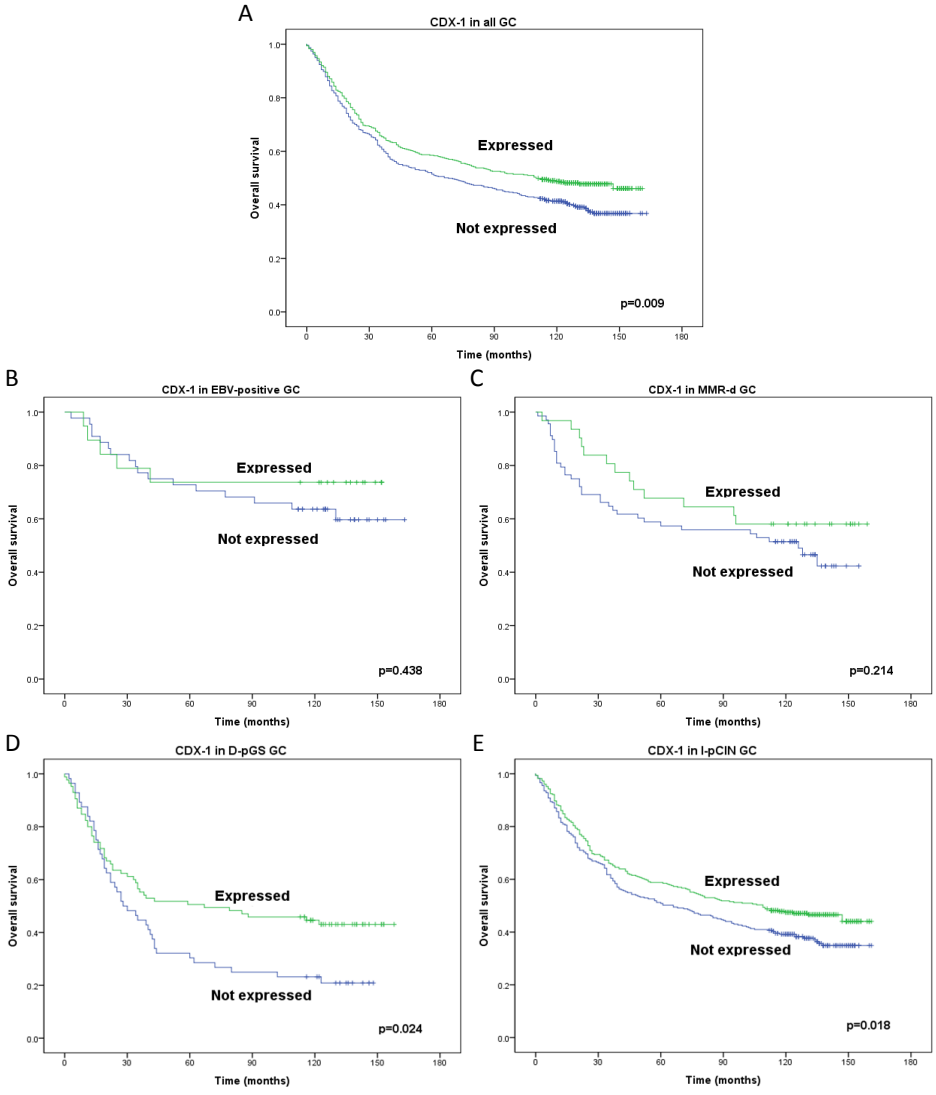


Figure 3: Comparison of overall survival according to CDX-1 expression
A, D, E: The groups expressing CDX-1 showed a favorable prognosis in D-pGS, I-pCIN and all GC groups. B, C: Overall survival according to CDX-1 expression. There is no significant survival difference between EBV-positive, MMR-d GC groups.

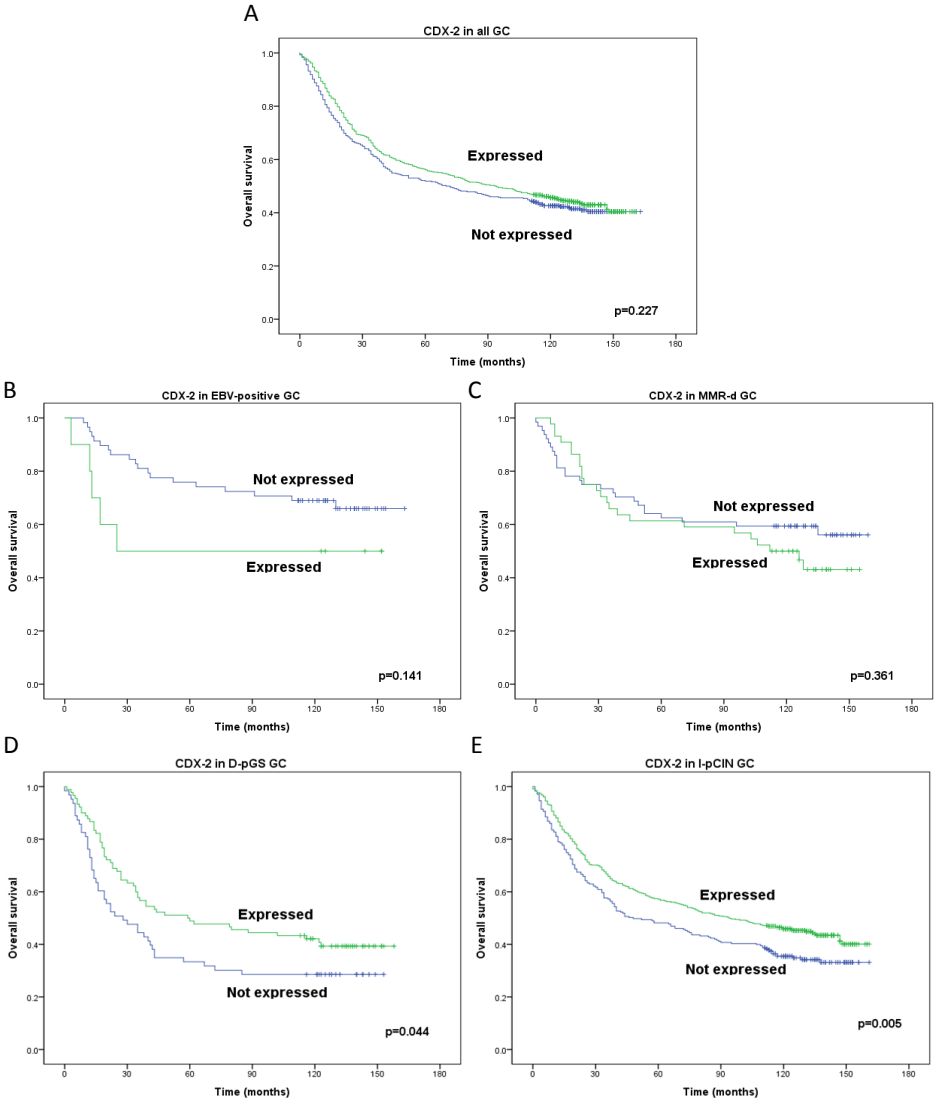


Figure 4: Comparison of overall survival according to CDX-2 expression
A, B, C: Overall survival according to CDX-2 expression. There is no significant survival difference in EBV-positive, MMR-d, and all GC groups. D, E: Overall survival according to CDX-2 expression. The groups expressing CDX-2 showed a favorable prognosis in D-pGS and I-pCIN GC groups..

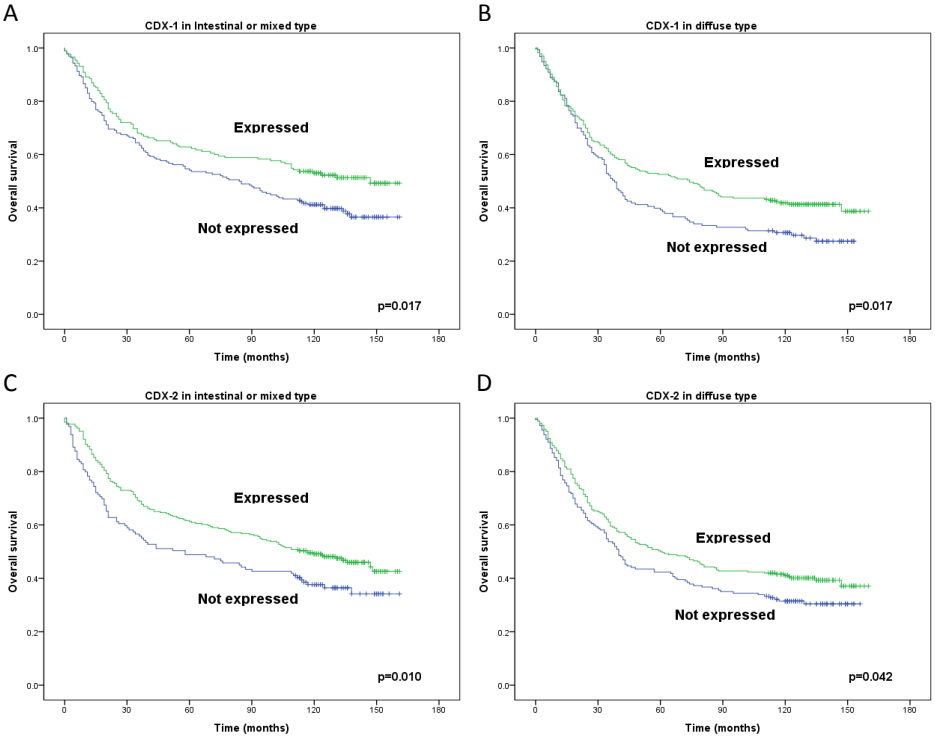


Figure 5: Comparison of overall survival according to the Lauren classification

A-B and C-D. Overall survival according to CDX-1 and CDX-2 expression in the Lauren classification. Expressed groups showed significantly favorable prognosis.

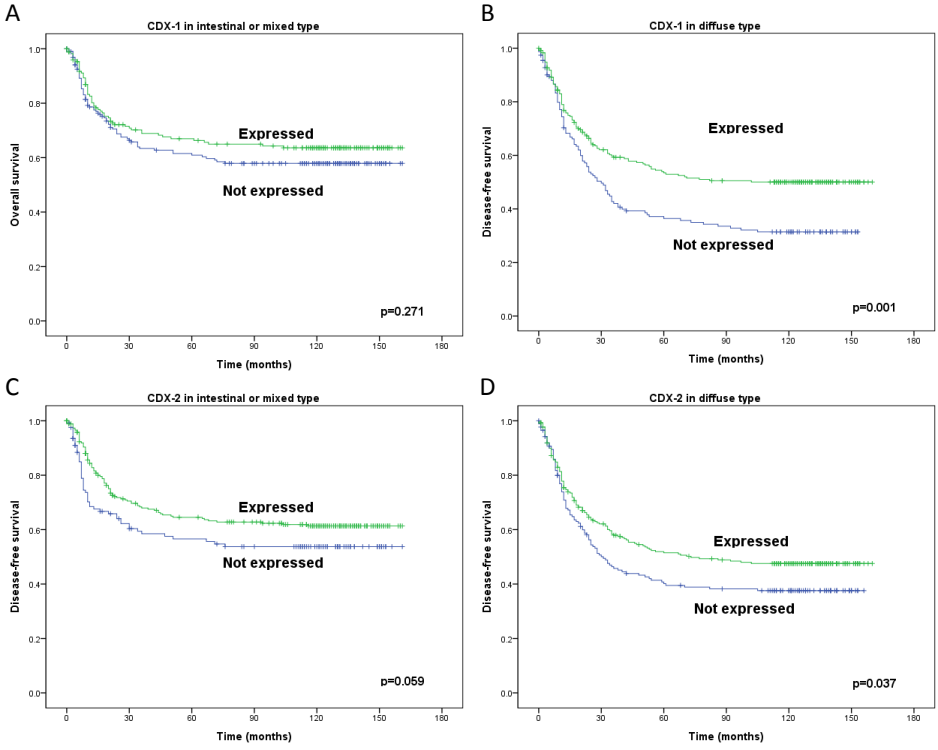


Figure 6: Comparison of disease-free survival according to the Lauren classification

A-B: Disease-free survival according to CDX-1 expression in the Lauren classification. The expressed group of the diffuse type showed a significantly better prognosis. C-D: Disease-free survival according to CDX-2 expression in the Lauren classification. The group expressing the diffuse type showed a significantly better prognosis.

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ABSTRACT(IN KOREAN)

위암에서 예후인자로서 Wnt 신호전달계 연관유전자의 발현분석

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김경민

서론
위암은 조직학적, 생물학적 특성이 매우 다양한 질병으로 최근 분자연구상 크게 4가지 그룹으로 분류되었고, 더 나아가 Wnt/β-catenin 신호전달계와 연관되어 있다는 것이 알려져 있다. 그러나 현재 위암에서 Wnt 연관 단백질의 발현과 예후의 관계에 관한 연구는 부족한 실정이다.
재료 및 방법
진행성위암 1158 케이스의 면역조직화학염색을 tissue microarray 에서 시행하였다. 사용한 항체는 CDX-1, CDX-2, XPNPEP3, TRIM24, Glutamine synthetase (GS), BMP-4, AXIN2, TCF4 이다..
결과
전체 환자 중 69 (5.9%) 명은 EBV 양성, 113 (9.7%) 명은 현미부수체불안정성,,나머지 978 (84.3%) 명이 EBV음성 현미부수체 안정성 위암이었다. EBV양성 현미부수체 불안정성 위암에서 CDX-1, CDX-2의 발현은 환자의 예후와 관계 없었다. 978명의 나머지 환자군에서 537 (54.9%) 명과 418 (42.7%) 명의 환자에서 CDX-2 와 CDX-1 이 발현되었다. 생존분석에서, CDX-1 와 CDX-2 발현은 환자의 좋은 예후와 유의하게 연관이 있었다. (다변량분석 p value=0.018 와 0.028). 그러나 다른 Wnt 연관 단백질, XPNPEP, TRIM24, GS, BMP-4, AXIN2, TCF4 에서는 통계적으로 유의하지 않았다. CDX-2 와 CDX-1 발현된 경우 Lauren 분류상 장형+혼합형, 미만형의 EBV음성 현미부수체안정성 위암에서 모두 좋은 생존율을 보였다 (log rank p; 0.010, 0.042 와 0.017, 0.017).
결론
CDX-1 및 CDX-2 의 발현은 EBV음성 현미부수체안정성 진행성 위암에서 좋은 예후인자이다

핵심되는 말 : CDX-1, CDX-2, gastric cancer, Wnt/β-catenin signaling pathway