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Frequent loss of heterozygosity of *SMAD4* locus and prognostic impacts of SMAD4 immunohistochemistry in gastric adenocarcinoma with enteroblastic differentiation

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Running head: Clinicopathologic impacts of *SMAD4* inactivation in GAED

Abstract

Gastric adenocarcinoma with enteroblastic differentiation (GAED) is a rare variant of gastric adenocarcinoma. Clinicopathologically, GAED is known to be aggressive and is characterized by frequent vascular invasion, lymphatic invasion, and liver metastasis even in early stages. *SMAD4* was identified as a frequently deleted gene in GAED by copy number variation analysis in our previous next-generation sequencing study; therefore, we examined the clinicopathological impacts of SMAD4 in 51 cases of GAEDs (early: 17, advanced: 34). We performed Sanger sequencing for *SMAD4* mutations and loss of heterozygosity (LOH) analysis of the *SMAD4* locus, in addition to immunohistochemistry for SMAD4, to determine its clinicopathological correlations and impacts on survival. The frequency of LOH at the *SMAD4* locus was 45.1%, and it was significantly higher in GAED compared to in conventional gastric adenocarcinoma. *SMAD4* mutations were not found in any case. Reduced SMAD4 expression was found in 60.8% of cases; it was significantly correlated with advanced stages and lymph node metastasis and showed trends of larger tumor size and lymphatic invasion. Reduced SMAD4 expression in metastatic lymph nodes was found in 21 of 36 cases. Survival analysis revealed that reduced *SMAD4* expression significantly affected the patient's overall survival (OS) and recurrence-free survival (RFS), although multivariate analysis showed that only liver metastasis and lymphatic infiltration (Ly+) were independent prognostic factors for OS and RFS. The *SMAD4* locus is one of the susceptibility genes in this

tumor, although *SMAD4* mutation was not detected. Furthermore, the inactivation of *SMAD4* appeared to contribute to the aggressiveness of GAED.

Keywords: Gastric adenocarcinoma; enteroblastic differentiation; SMAD4; loss of heterozygosity

Introduction

Gastric adenocarcinoma with enteroblastic differentiation (GAED) is a rare variant of gastric adenocarcinoma characterized by fetal gut-like structures with glycogen-rich clear cytoplasm and partially overlaps with alpha-fetoprotein (AFP)-producing gastric cancer (AFP-GC) [1-3]. Histologically, this tumor is composed of cuboidal or columnar cells with clear cytoplasm and a tubulo-papillary or solid sheet-like growth pattern, and is accompanied by conventional adenocarcinoma in most cases [3-5]. Furthermore, oncofetal proteins, such as AFP, glypican-3 (GPC3) and spalt-like transcription factor 4 (SALL4), are known immunohistochemical staining markers for GAED [6, 7]. Clinicopathologically, we previously reported that GAED shows aggressive behavior characterized by frequent lymphovascular invasion, as well as lymph node and liver metastasis even in early cancer, compared to in conventional gastric adenocarcinoma (CGA) [8, 9].

We recently found that GAED has a high frequency of *TP53* mutations associated with p53 overexpression by comprehensive analysis using next-generation sequencing (NGS). Furthermore, copy number variation (CNV) analysis revealed that *ERBB2* amplification was the most frequent alteration in GAED, and we found that trastuzumab may be effective for GAED as well as conventional gastric adenocarcinoma [10].

SMAD4 is a tumor suppressor gene involved in the transforming growth factor-β signaling pathway, which is involved in various developmental processes, such as morphogenesis,

cellular proliferation, and tumorigenesis. *SMAD4* was originally identified on chromosome 18q21.2 in pancreatic ductal adenocarcinoma [11]. In pancreatic and colorectal cancer, inactivation of *SMAD4* through the loss of heterozygosity (LOH) or gene mutation is frequently observed and associated with tumor progression [12]. In contrast, LOH and mutation of *SMAD4* show a weaker relationship with the loss of SMAD4 expression in brain, head and neck, lung, breast, esophageal, gastric, uterine, prostate, renal, and bladder tumors [12, 13]. However, reduced *SMAD4* expression has been shown to be associated with tumor progression and poor prognosis in several tumors in previous studies [14]. Interestingly, *SMAD4* was identified as a frequently deleted gene in GAED through CNV analysis using NGS, indicating that SMAD4 plays an important role in the tumorigenesis or tumor progression of GAED [10].

This study was conducted to determine the significance of *SMAD4* in GAED by evaluating the frequency and prognostic impacts of SMAD4 expression, *SMAD4* mutation, and *SMAD4* LOH.

2. Materials and Methods

2.1. Case selection

We used a series of 51 GAED cases evaluated in our recent study [10]. Briefly, GAED was defined as a tumor with a predominant adenocarcinoma component and clear cytoplasm similar to in the fetal gut, growing as a tubulo-papillary or solid sheet-like structure, and with more than 10% immunohistochemical positivity for AFP, GPC3, or SALL4. The 51 GAED cases consisted of 17 cases with early gastric cancer and 34 with advanced gastric cancer. All patients were followed-up every 3 months after surgery. The survival periods were determined as survival time after diagnosis. The mean follow-up time for patients with GAED was 39.2 months (range, 2–108 months). This study was reviewed and approved by the Juntendo University School of Medicine Institutional Review Board (#2016107).

2.2. Immunohistochemistry

Immunohistochemistry analyses were performed on 4-mm formalin-fixed paraffin embedded tumor tissues. We evaluated *SMAD4* because it was identified as one of the frequently deleted genes in GAED by CNV analysis in our previous NGS study [10]. Immunohistochemical analysis of SMAD4 expression was performed for 51 GAED tissues and the corresponding metastatic lymph nodes for 36 cases. We used a mouse monoclonal SMAD4 antibody (1:50, Santa Cruz Biotechnology, Dallas, TX, USA) for detection. Immunohistochemical staining results were evaluated by two pathologists (T.S. and N.Y.).

When discrepancies arose, the cases were reviewed using a multiheaded microscope to reach a consensus. *SMAD4* expression in tumor cells was evaluated only in the GAED area within the tumor to determine the significance of SMAD4 expression and enteroblastic differentiation of this tumor. Expression levels were compared with those of normal epithelial cells within the slide. Only nuclear staining was evaluated, and nuclear and diffuse (>50% of GAED area) staining in the tumor showing the same intensity as in the normal epithelium was considered as a positive. In contrast, samples showing weaker staining in normal epithelial cells or no staining were considered as negative. Tumors were then classified according to their expression of *SMAD4* upon evaluation of the section, with "preserved expression" considered as >50% of the tumor cells showing positive. All other tumors were classified as having "reduced expression".

2.3. Sanger sequencing

Mutations in *SMAD4* were evaluated by Sanger sequencing for all 51 GAED cases including 24 cases that were examined in our previous NGS study using genomic DNA in polymerase chain reaction (PCR), followed by direct sequencing. Genomic DNA was extracted described as previously [10]. Nine primer pairs listed on the Cancer hotspot panel were used (Supplementary Table 1). PCR products were cut from the gel and purified by PCR clean-up Gel extraction (MACHEREY-NAGEL, Düren, Germany). Purified PCR products were sequenced with dideoxynucleotides (BigDye Terminator v3.1; Applied Biosystems, Foster

City, CA, USA) and specific primers, purified using a BigDye X Terminator Purification Kit (Applied Biosystems), and analyzed with a capillary sequencing machine (3730xl Genetic Analyzer; Applied Biosystems). Sequences were then examined with Sequencing Analysis version 3.5.1 software (Applied Biosystems). Mutations were detected by identifying cases in which the height of the mutated peak reached 20% of the height of the normal peak through capillary electrophoresis. All mutations were verified by sequencing of the sense and antisense strands. Mutations were evaluated by 2 independent researchers (N.Y. and T.S.).

2.4. Loss of heterozygosity analysis

LOH analysis at the *SMAD4* locus was performed in all cases. Six polymorphic microsatellite markers at the *SMAD4* locus (D18S845, D18S1110, D18S474, D18S69, D18S74E, D18S851) were used (Supplementary Table 1) [15, 16]. The amplified PCR products were evaluated with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). LOH was determined as previously described [17, 18]. Briefly, cases showing an allelic imbalance factor greater than 1.5 or less than 0.5 for at least one marker were considered to show LOH. The LOH rate at the *SMAD4* locus in GAED was compared to the deletion rate of *SMAD4* in The Cancer Genome Atlas (TCGA) database [19].

2.5. Survival analysis and statistical analysis

Correlations between clinicopathological factors and SMAD4 expression were analyzed by

χ^2-test or Fisher's exact test. The deletion rate of *SMAD4* in CGA was obtained from the TCGA database. Correlations between the LOH rate at the *SMAD4* locus in GAED and the deletion rate of *SMAD4* in CGA obtained from the TCGA database were analyzed using Fisher's exact test. To determine the prognostic impact of SMAD4 expression and LOH, we performed Kaplan-Meier survival analysis and log-rank tests. A *P* value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Immunohistochemistry for SMAD4 and its clinicopathological impacts in GAED

Table 1 summarizes the results of SMAD4 immunohistochemistry analysis of 51 cases of GAED. Most cases considered as preserved expression showed diffuse staining patterns. Reduced SMAD4 expression in GAED (Fig. 1) was observed in 31 cases (60.8%), and clinicopathological factors were described in our previous study [10]. Reduced SMAD4 expression in GAED was significantly correlated with advanced stage (p = 0.01) and lymph node metastasis (p = 0.01). Reduced SMAD4 expression in GAED was also correlated with a larger tumor size (p = 0.07) and lymphatic invasion (p = 0.07), although the trend was not significant (Table 2). LOH at the *SMAD4* locus was not associated with reduced SMAD4 expression. Furthermore, the status of SMAD4 expression in the corresponding metastatic lymph nodes was assessed. Reduced SMAD4 expression in corresponding lymph nodes was detected in 21 of 36 cases (58.3%). Lymph node metastasis and reduced SMAD4 expression in GAED were significantly correlated, whereas SMAD4 expression in metastatic lymph nodes was not necessarily reduced.

3.2. Mutation analysis

Sanger sequencing of *SMAD4* was performed for all GAED cases. *SMAD4* mutation was not found in any case. In the TCGA database, the *SMAD4* mutation in CGA was observed in

8.3% of cases (24/290), but was not observed in GAED.

3.3. LOH analysis

Fifty-one GAED cases were analyzed for LOH at the *SMAD4* locus. We chose six polymorphic microsatellite markers (D18S845, D18S1110, D18S474, D18S69, D18S74E, D18S851). We found that the frequency of LOH at the *SMAD4* locus was 45.1% (23/51) (Fig. 2). Table 1 summarizes the SMAD4 genetic alterations in 51 cases of GAED. Based on the TCGA database [19], the frequency of LOH at the *SMAD4* locus was significantly higher in GAED than in CGA (23/51, 45.1% vs. 18/295, 6.1%, $p < 0.01$).

3.4. Prognosis in GAED

We updated the prognostic data from our previous report [10]. The 3-year overall survival (OS) rate in GAED was updated to 53.9% and 3-year recurrence free survival (RFS) rate was 44.9% (Fig. 3A, B). Survival analysis revealed that the 3-year OS was significantly worse for patients with reduced SMAD4 expression ($p = 0.040$) in GAED; additionally, the 3-year RFS also was significantly worse for the same group of patients ($p = 0.047$) (Fig. 3C, D). Survival analysis was also performed for the stratified groups (only early cases and only advanced cases). In early GAED group, cases with preserved SMAD4 expression showed better prognosis than those with reduced SMAD4 expression, though not being statistically significant (Fig. 3E, F). In the advanced GAED, prognostic impact of SMAD4 was lost (Fig. 3G, H). LOH status at the *SMAD4* genetic locus was not associated with OS and RFS.

Furthermore, multivariate analysis showed that liver metastasis and lymphatic infiltration were independent prognostic factors for both overall OS ($p < 0.001$, $p = 0.006$) and RFS ($p < 0.001$, $p = 0.015$).

Discussion

SMAD4 on chromosome 18q21.2 is a tumor suppressor gene that mediates the transforming growth factor-β signaling pathway. Previous studies have shown that inactivation of *SMAD4* plays an important role in the tumorigenesis and progression of pancreatic, colorectal, gastric, esophageal, breast, lung, cervical, endometrial cancer, bile duct, and other solid tumors [14, 20-28]. Reduced SMAD4 expression was found to be correlated with a larger tumor size, lymphatic invasion, and lymph node metastasis in pancreatic cancer [20]; lymphovascular infiltration and lymph node and liver metastasis in colorectal cancer [21, 29], lymph node and liver metastasis in bile duct cancer [28]; and lymph node metastasis in esophageal, breast, and lung cancers [23, 30, 31]. Another previous study reported that reduced SMAD4 expression was significantly correlated with lymph node metastasis in CGA [22]. However, the mechanism of tumor progression associated with *SMAD4* in GAED is not fully understood.

In this study, we assessed the frequency and prognostic impact of SMAD4 expression, mutation, and LOH, as *SMAD4* was identified as a frequently deleted gene through CNV analysis using NGS in our previous study [10]. Inactivation of *SMAD4* is common in pancreatic and colorectal cancers, and probably occurs through LOH and gene mutation (the two-hit hypothesis) [12]. Previous studies reported that *SMAD4* mutation was rare, whereas LOH was observed in 6.1–56% of CGA cases, although the cut-off for LOH in these

studies does not appear to be strict compared to the cut-off used by us in this study [19, 22, 32, 33]. In the present study, we did not detect *SMAD4* mutations in any of the evaluated cases, and LOH at the *SMAD4* locus was found in 23 cases (45.1%). Compared to the TCGA data, LOH at the *SMAD4* locus in GAED was significantly higher than that in CGA [19]. However, LOH at the *SMAD4* locus in GAED was not associated with reduced SMAD4 expression. It is generally considered that LOH alone is not enough to suppress SMAD4 expression [34] as two hits on both alleles are required to inactivate a tumor suppressor and cause cancer progression. Thus, LOH at the *SMAD4* locus may be partially involved in tumor progression in GAED, although other epigenetic changes may influence inactivation of this gene.

Reduced SMAD4 expression in GAED was significantly correlated with advanced stage and lymph node metastasis, and the same trends were observed for larger tumor size and lymphatic infiltration. As a comparison, we selected 152 sequential CGA cases of well to moderately differentiated types surgically resected from patients admitted at our hospital from January 2009 to December 2013 and performed immunostaining for SMAD4. Reduced SMAD4 expression in CGA was significantly correlated with patient age ($p = 0.03$), advanced stage ($p < 0.01$), liver metastasis ($p = 0.02$), lymphatic infiltration ($p = 0.04$), and lymph node metastasis ($p = 0.04$). Supplementary Table 2 summarizes the CGA patients' characteristics, and the impact of SMAD4 expression on clinicopathological factors is

summarized in Supplementary Table 3. Comparison of SMAD4 IHC between GAED and CGA revealed a similar trend in several points such as a correlation with advanced stage, lymphatic infiltration, and lymph node metastasis. However, a correlation between reduced SMAD4 expression and liver metastasis was observed only in CGA. Thus, it was considered that GAED caused liver metastasis in an earlier stage than CGA. Survival analysis using updated prognostic information indicated that reduced SMAD4 expression in GAED significantly affected the OS and RFS of the patients, whereas reduced SMAD4 expression in CGA did not affect OS and RFS (Supplementary Fig. 1). These results suggest that inactivation of *SMAD4* affects tumor progression and patient prognosis in GAED similarly to in previously mentioned cancers, although multivariate analysis failed to identify reduced SMAD4 expression as an independent prognostic factor. Fujii et al. reported that LOH at various loci was detected in AFP-GC, and LOH at 18q was detected on 58% of cases [35]. They also demonstrated that additional LOH of 13q may be involved in acquisition of the AFP-producing phenotype [35]. Our finding that reduced SMAD4 expression was less frequently observed in early GAED cases is, in part, consistent with these results, suggesting that *SMAD4* located on 18q is involved in the progression to invasive cancer rather than tumorigenesis of GAED. Thus, as our CNV data may include significant genes other than *SMAD4*, further research is necessary to provide insight into the tumorigenic process of this cancer.

Finally, SMAD4 expression in the corresponding metastatic lymph nodes was evaluated. Histologically, metastatic tumors in the lymph nodes were mainly composed of the GAED component, occasionally accompanied by a CGA area. However, SMAD4 expression in metastatic lymph nodes was not necessarily reduced. These findings suggest that the epigenetic regulation of *SMAD4* is necessary for the process of metastasis.

In conclusion, the LOH rate (45.1%) of the *SMAD4* locus in GAED was significantly higher than that of CGA, suggesting that this locus a susceptibility gene in this tumor. Furthermore, the inactivation of *SMAD4* appeared to contribute to the acquisition of the aggressive behavior of GAED.

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Figure legends

Figure 1.

SMAD4 immunohistochemical staining in gastric adenocarcinoma with enteroblastic differentiation (GAED). A, B: Hematoxylin and eosin staining of GAED tissue. Reduced SMAD4 expression was observed in a case of GAED (C: corresponding to A). Preservation of SMAD4 expression was observed in a case of GAED (D: corresponding to B). Original magnification (A–D): ×200.

Figure 2.

A, B: Loss of the long allele was observed in a tumor sample (B) compared to in the corresponding normal tissue (A), indicating loss of heterozygosity (LOH) (D18S851). C: LOH rates at the *SMAD4* locus in gastric adenocarcinoma with enteroblastic differentiation (GAED) was 45.1%. Referred to The Cancer Genome Atlas database, LOH rates at *SMAD4* locus in GAED was significantly higher than that in conventional gastric adenocarcinoma (CGA). $*p < 0.01$.

Figure 3

Kaplan-Meier survival curves. A: The 3-year overall survival (OS) rate for this series of gastric adenocarcinoma with enteroblastic differentiation (GAED) is 53.9%. B: The 3-year recurrence-free survival (RFS) rate for this series of gastric adenocarcinoma with GAED is 44.9%. C, D: OS and RFS rates according to the SMAD4 expression in GAED. Group with

preserved SMAD4 expression showed better overall survival rate (C) and recurrence-free survival rate (D) compared to that with reduced expression. E, F: OS (E) and RFS (F) rates according to the SMAD4 expression only in early GAED cases. In this group, cases with preserved SMAD4 expression showed better prognosis than those with reduced SMAD4 expression, though not being statistically significant. G, H: OS (G) and RFS (H) rates according to the SMAD4 expression only in advanced GAED cases. In the advanced cases, prognostic impact of SMAD4 was lost.

(+): Preserved expression, (-): Reduced expression.

Supplementary Figure legends

Supplementary Figure 1: Overall survival (OS) (A) and recurrence free survival (RFS) (B) rates for control conventional gastric adenocarcinoma (CGA) in this study. Reduced SMAD4 expression in CGA did not affect OS (C) and RFS (D), although cases with reduced SMAD4 expression showed worse prognosis than those with preserved SMAD4 expression.

Table 1. Association between LOH at *SMAD4* locus and SMAD4 immunohistochemistry in GAED

Case	Loss of *SMAD4* by CNV	Markers at *SMAD4* locus						Reduced SMAD4 IHC
		D18S845	D18S1110	D18S474	D18S69	D18S74E	D18S851	
1	NA	+	-	-	+	+	-	+
2	NA	+	-	-	-	+	+	+
3	NA	+	-	-	-	-	-	-
4	NA	-	-	-	-	+	+	-
5	NA	+	+	-	+	-	-	-
6	NA	-	-	-	-	-	-	-
7	NA	-	-	-	-	-	-	-
8	NA	+	+	-	-	-	+	-
9	NA	+	-	-	-	+	+	-
10	NA	-	-	-	-	-	-	-
11	NA	-	-	-	-	-	-	-
12	NA	-	-	-	-	-	-	+
13	NA	-	-	-	-	-	-	-
14	NA	+	-	+	-	+	+	+
15	NA	-	+	-	-	-	-	-
16	-	-	-	-	-	+	-	+
17	-	-	-	-	-	-	-	-
18	NA	-	-	-	-	-	-	+
19	NA	-	-	-	-	-	-	+
20	NA	+	-	-	-	-	-	+
21	NA	-	-	-	-	-	-	+
22	NA	-	-	-	-	-	-	+
23	NA	-	-	-	-	-	+	-
24	NA	-	-	-	-	-	-	+
25	-	-	-	-	-	-	-	+
26	NA	-	-	-	-	-	-	+
27	NA	-	-	-	-	+	-	+
28	NA	-	-	-	-	-	-	+
29	-	-	-	-	-	-	-	+
30	NA	-	-	-	+	+	-	+
31	-	-	-	-	-	-	-	-
32	-	-	+	-	-	-	-	-

33	+	+	-	-	-	-	+	+
34	+	+	-	-	-	-	-	+
35	-	-	-	-	-	+	+	+
36	NA	-	-	-	-	-	-	+
37	+	-	-	-	-	-	-	-
38	+	-	-	-	-	-	-	+
39	-	-	-	-	-	-	-	+
40	+	-	-	-	-	+	+	-
41	-	-	-	-	-	-	-	+
42	-	+	-	-	-	-	-	-
43	+	-	-	-	-	-	-	+
44	-	-	-	-	-	-	-	-
45	-	-	-	-	-	-	-	-
46	-	+	-	-	-	-	+	+
47	-	-	-	-	-	-	-	+
48	-	-	-	-	-	-	+	+
49	-	-	-	-	-	-	-	+
50	-	+	-	-	-	-	-	+
51	-	-	-	-	-	-	-	+

Abbreviation: CNV, Copy number valiation.

Table 2. Association between SMAD4 reduced expression and clinicopathological factors in 51 GAED cases.

	SMAD4 reduced expression (+) n=31	SMAD4 reduced expression (-) n=20	p value
Age (years) (mean ± SD)	70.0±10.1	72.9±7.55	0.25
Gender (male/female)	27/4	15/5	0.29
Tumor location (Upper/Middle/Lower)	10/8/13	5/4/11	0.71
Tumor size (mm) (mean ± SD)	50.9±32.1	30.5±27.4	0.07
Therapy method (Operation/ESD)	30/1	14/6	0.01
Macroscopic type for early cancer (elevated/depressed)	2/4	1/11	0.25
Macroscopic type for advanced cancer (Type 1/2/3/4/5)	2/13/9/0/1	0/7/1/0/0	0.48
Invasion depth (early/advanced)	5/26	12/8	<0.01
TNM stage (pStage 1/ pStage 2, 3, 4)	4/27	11/9	<0.01
Lymphatic invasion (+)	24	10	0.07
Venous invasion (+)	20	15	0.54
Lymph nodes metastasis (+)	27	9	<0.01
Liver metastasis (+)	13	4	0.12
Growth patterns (Sllid/Tubulo-papillary type)	10/21	6/14	1
Immunohistochemical analysis			
AFP (+)	9	7	0.76
GPC3 (+)	25	17	1
SALL-4 (+)	25	16	1

Abbreviation: GAED, gastric adenocarcinoma with enteroblastic differentiation; SD, standard deviation.

Highlights

・LOH at *SMAD4* Locus was frequently observed in GAED and *SMAD4* locus was considered to be one of the susceptibility genes, although *SMAD4* mutation was absent in GAED.

・Reduced *SMAD4* expression significantly affected the patient's overall and recurrence free survival in GAED.

・Inactivation of *SMAD4* seemed to contribute to the acquisition of aggressive behavior such as larger tumor size and advanced stage in GAED.

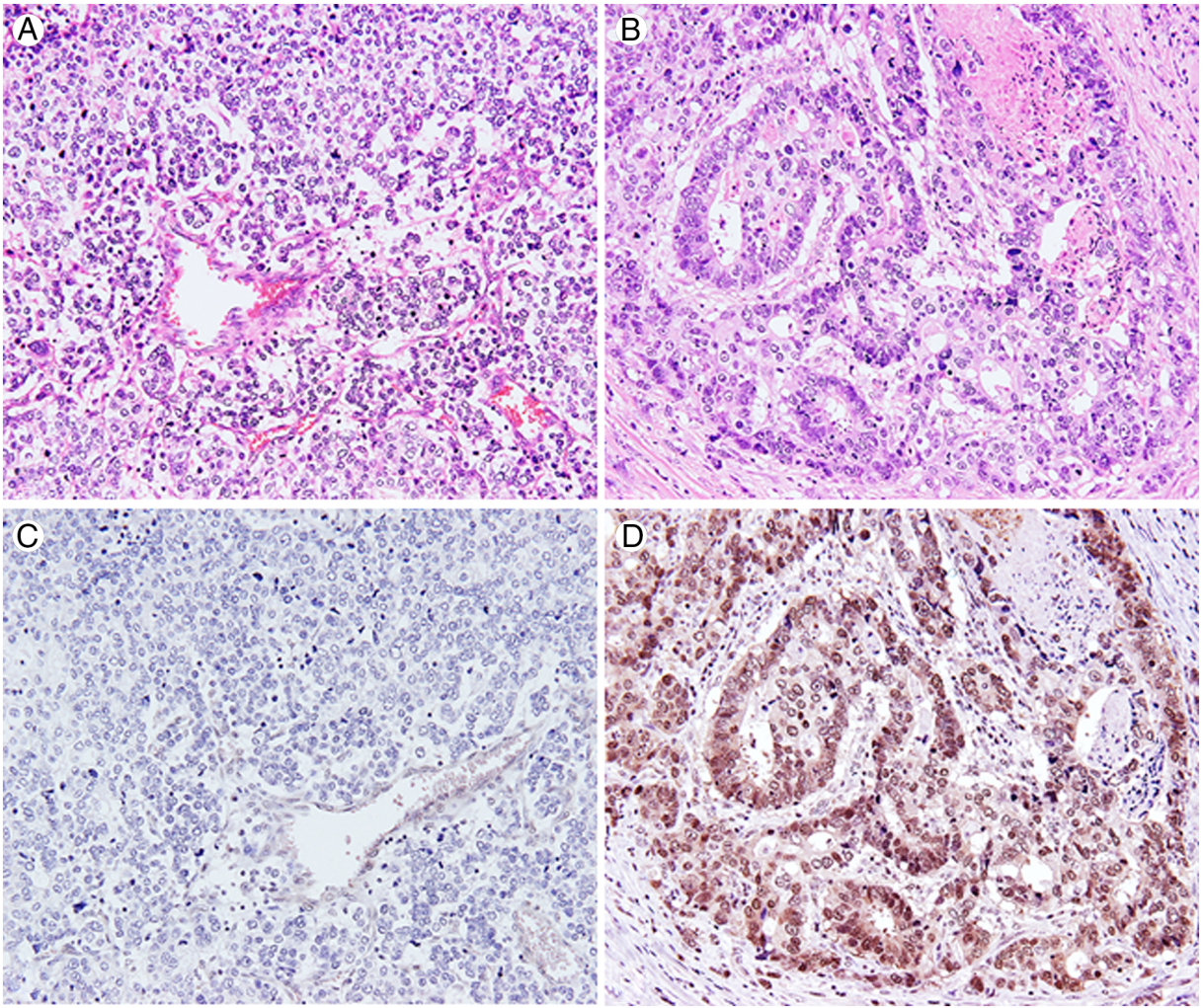

Figure 1

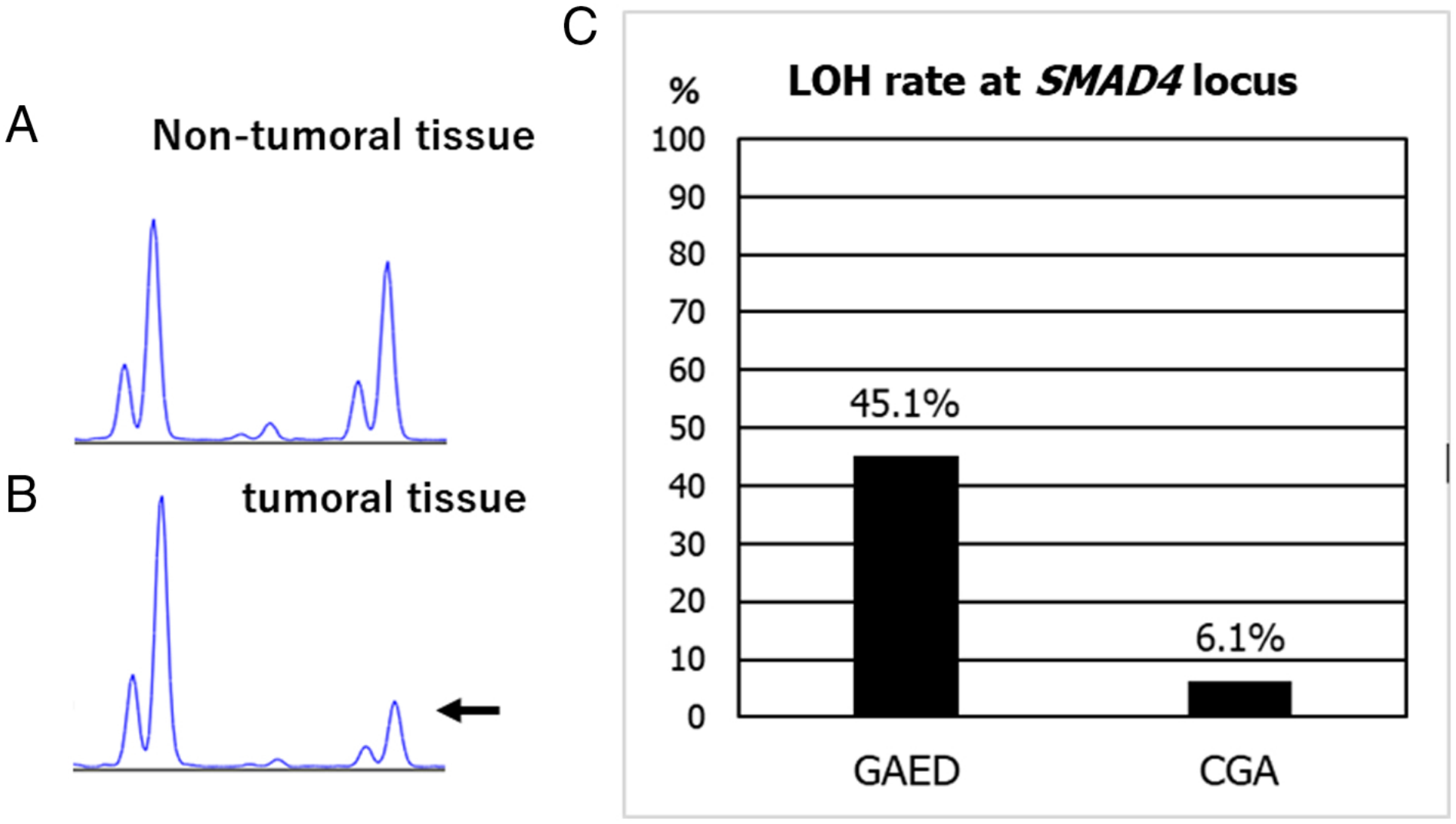
A
Non-tumoral tissue
B
tumoral tissue
C
LOH rate at SMAD4 locus
%
100
90
80
70
60
50
40
30
20
10
0
45.1%
6.1%
GAED
CGA

Figure 2

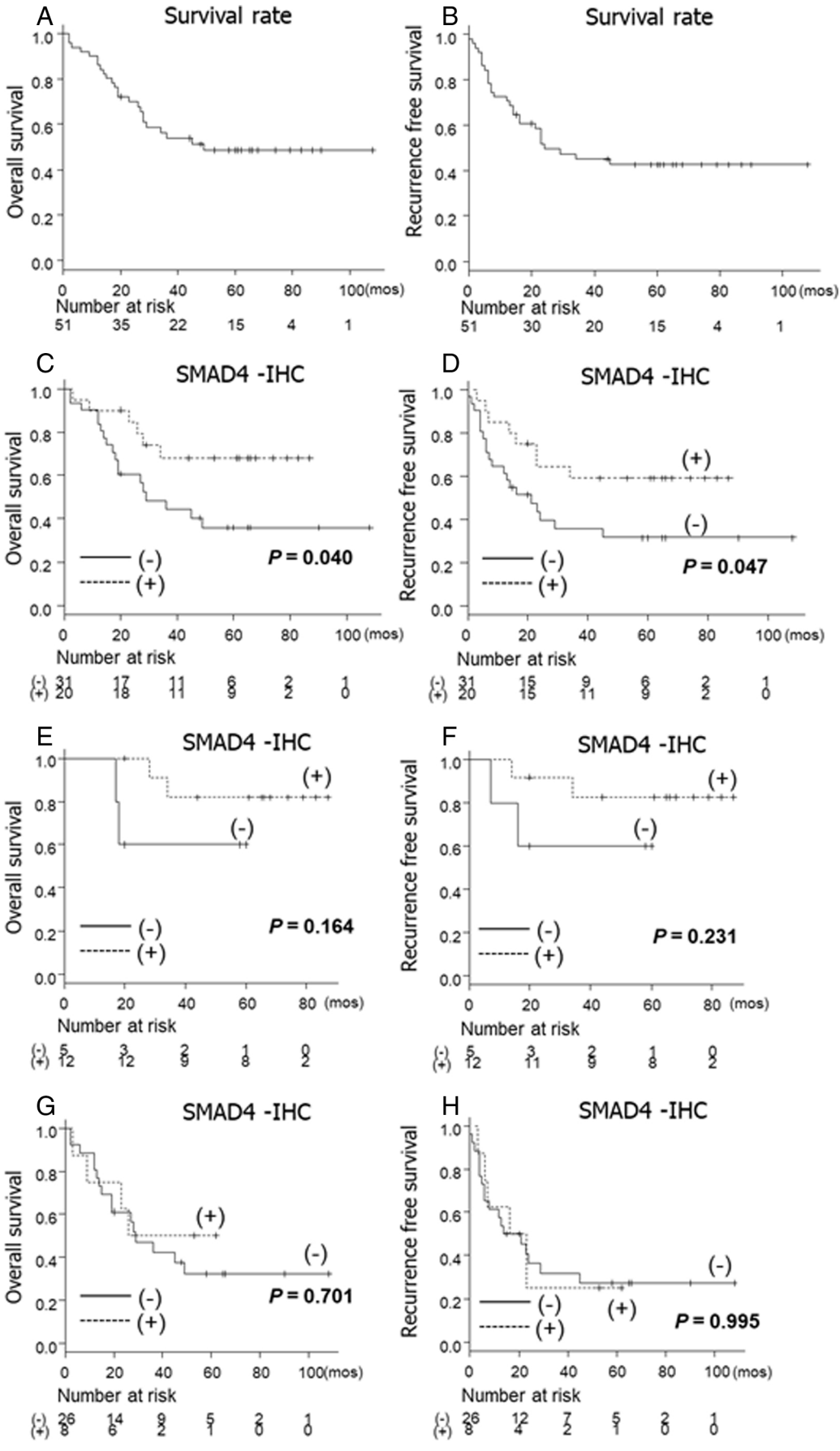
A
Survival rate
Overall survival
1.0
0.8
0.6
0.4
0.2
0.0
0 20 40 60 80 100(mos)
Number at risk
51 35 22 15 4 1
B
Survival rate
Recurrence free survival
1.0
0.8
0.6
0.4
0.2
0.0
0 20 40 60 80 100 (mos)
Number at risk
51 30 20 15 4 1
C
SMAD4 -IHC
Overall survival
(-)
(+)
P = 0.040
0 20 40 60 80 100 (mos)
Number at risk
(-) 31 17 11 6 2 1
(+) 20 18 11 9 2 0
D
SMAD4 -IHC
Recurrence free survival
(+)
(-)
(-)
(+)
P = 0.047
0 20 40 60 80 100 (mos)
Number at risk
(-) 31 15 9 6 2 1
(+) 20 15 11 9 2 0
E
SMAD4 -IHC
Overall survival
(+)
(-)
(-)
(+)
P = 0.164
0 20 40 60 80 (mos)
Number at risk
(-) 5 3 2 1 0
(+) 12 12 9 8 2
F
SMAD4 -IHC
Recurrence free survival
(+)
(-)
(-)
(+)
P = 0.231
0 20 40 60 80 (mos)
Number at risk
(-) 5 3 2 1 0
(+) 12 11 9 8 2
G
SMAD4 -IHC
Overall survival
(+)
(-)
(-)
(+)
P = 0.701
0 20 40 60 80 100(mos)
Number at risk
(-) 26 14 9 5 2 1
(+) 8 6 2 1 0 0
H
SMAD4 -IHC
Recurrence free survival
(-)
(-)
(+)
(+)
P = 0.995
0 20 40 60 80 100 (mos)
Number at risk
(-) 26 12 7 5 2 1
(+) 8 4 2 1 0 0

Figure 3

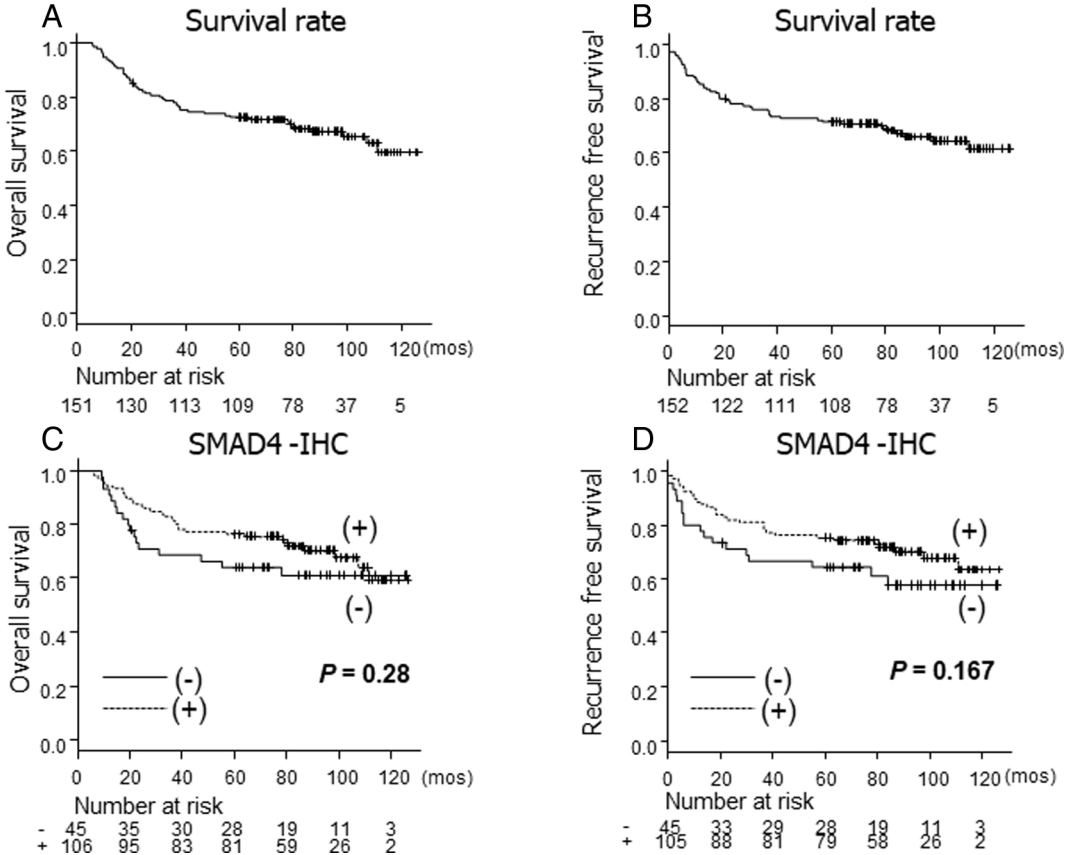
A
Survival rate
Overall survival
1.0
0.8
0.6
0.4
0.2
0.0
0 20 40 60 80 100 120 (mos)
Number at risk
151 130 113 109 78 37 5
B
Survival rate
Recurrence free survival
1.0
0.8
0.6
0.4
0.2
0.0
0 20 40 60 80 100 120 (mos)
Number at risk
152 122 111 108 78 37 5
C
SMAD4 -IHC
Overall survival
(+)
(-)
(-)
(+)
P = 0.28
0 20 40 60 80 100 120 (mos)
Number at risk
- 45 35 30 28 19 11 3
+ 106 95 83 81 59 26 2
D
SMAD4 -IHC
Recurrence free survival
(+)
(-)
(-)
(+)
P = 0.167
0 20 40 60 80 100 120 (mos)
Number at risk
- 45 33 29 28 19 11 3
+ 105 88 81 79 58 26 2

Figure 4