The prognostic significance of Plakophilin-1 expression in esophageal cancer

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Running title: Plakophilin 1 in esophageal cancer

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Abstract

Background/Aim: This study evaluated whether PKP1 is a prognostic marker for esophageal cancer (EC).

Materials and Methods: We tested immunohistochemically for PKP1 in squamous cell carcinoma EC specimens from 99 patients, including cytoplasmic (C), membrane (M), and nuclear (N) cellular areas, and analyzed their relationships with clinicopathological factors.

Results: PKP1stains were stratified into strong and weak for all three cellular areas. Staining was inversely related to tumor depth (C: *P*=0.002, M: *P*=0.00007, N: *P*=0.02), lymph node metastasis (C: *P*=0.003, M: *P*=0.001, N: *P*=0.004) and pathological stage (C: *P*=0.0004, M: *P*=0.0001, N: *P*=0.006). Cytoplasmic and membrane staining were inversely related to vessel invasion. Patients with strong C stain had better overall survival than those with weak C stains (*P*=0.01). Disease-free survival of patients with strong M stains was better than that of those with weak staining (*P*=0.01).

Conclusions: Cytoplasmic and membrane PKP1 expression is a possible prognostic marker for EC.

Introduction

Esophageal cancer (EC) cases have been gradually increasing as the population's median age increases. EC is one of the 10 most common cancers and is responsible for more than half a million deaths worldwide (1).

In Japan, approximately 14,000 treated cases of EC are reported annually by the Japanese Society of Thoracic Surgery (2). Although the percentage of adenocarcinomas among lower ECs has been increasing, more than 90% of EC in Japan is still squamous cell carcinoma. These cancers generally occur in elderly individuals who both smoke and drink alcohol and who have a high risk of developing other cancers, such as in the aerodigestive tract (3). The esophagus also has abundant lymphatic tissue in the lamina propria and the submucosa, so that lymph nodes can easily metastasize. Therefore, although advances in diagnosis and treatment have improved EC outcomes, EC is still considered to have a poor prognosis (4). Elucidation of EC carcinogenesis and identification of new biomarkers and therapeutic targets could improve this dismal pattern (5).

Changes in cell–cell adhesion are associated with tumor dedifferentiation and invasion. Plakophilins (PKP1, PKP2, and PKP3) are in the armadillo-like protein subfamily and are related to p120 (6). PKP1 is an important plaque component of major intercellular

adhesion junctions, which act as fixation points for desmosomes, that is, intermediate filaments. Strong expression of PKP1, inhibits cell proliferation, colony formation, migration, and invasion, and enhances apoptosis. Its expression is inversely related to cancer grade (4). Although strong PKP expression has been observed in several cancers (e.g., pharyngeal, colorectal, lung, and ovarian), no studies of its role in esophageal SCC have been reported so far. This study therefore investigated PKP1's expression and clinical significance in EC.

Materials and Methods

Study Population

The subjects for this study were 99 patients who underwent radical resection for EC at Dokkyo Medical University Hospital from May 1, 2009 to December 31, 2018. We excluded patients who received preoperative chemotherapy. This study was approved by the Ethics Committee of Dokkyo Medical University Hospital (No: R-24-11J). Its methods and experimental protocols were carried out in accordance with the approved guidelines of Dokkyo Medical University Hospital. All patients gave written informed consent, obtained preoperatively, for use of their specimens.

Immunohistochemistry

Samples were fixed in buffered formalin and embedded in paraffin blocks from which 3–4-μm sections were cut for conventional hematoxylin and eosin staining (7). These sections were harvested from paraffin blocks, mounted on pretreated slices and stained, using the VECTASTAIN Elite ABC Rabbit IgG Kit (PK-6101). Normal human esophageal tissue was used as a positive control. Several dilutions were tested; finally, we used a dilution of 1:200 (7). Sections were deparaffinized; for antigen retrieval the sections were heated in sodium citrate buffer (pH 6.0; LSI Medience RM102-C) at 95°C for 20 minutes in a microwave. They were cooled for 60–70 min until they returned to room temperature and were washed with water for 5 min. Next, they were treated with 0.3% hydrogen peroxide methanol for 30 min to elicit endogenous peroxidase activity and again washed with water for 5 min, then blocked with goat normal serum (Vectastain Elite ABC Rabbit IgG Kit) for 30 min at room temperature. Sections were incubated with primary antibodies for 60 minutes at room temperature and washed three times with phosphate-buffered saline (PBS) for 5 minutes, then incubated with secondary antibodies at room temperature for 30 minutes and washed three times with PBS for 5 minutes. Finally, they were nucleate stained with hematoxylin for 1 minute and rinsed in running water for 10 minutes.

Evaluation

We reported PKP+ cells as percentages of the total number of carcinoma cells. Immunostaining reactions were classified as homogeneous (50%–100%), focal (10%–50%), or negative (0%–10%); and further scored as 6 (strong brown), 5 (moderate yellow), or 4 (weak light yellow) among homogeneous samples, and 3 (strong brown), 2 (moderate yellow), or 1 (weak light yellow) among focal samples. A score of 0 indicated no staining; however, no samples in this study scored 0 (6). A score of 0–4 was considered weak and 5–6 was considered strong.

Statistical analysis

Statistical differences between categoric variables were determined using Fisher's exact test. Survival analysis was performed using the Kaplan–Meier method to evaluate survival time distribution.

Results

Patients' characteristics

For the 99 patients, mean age was 68.1±9.7 years. They included 86 men and 13 women. Their tumor locations in the esophagus were upper: *n*=14, middle: *n*=52, and lower: *n*=33 cases. Clinicopathological factors are shown in Table 1.

Immunostaining patterns

The PKP1 immunohistological results, along with positive controls, are shown in Figure 1. Although PKP1 expression is generally more confined to the nucleus than in the cytoplasm in adenocarcinoma, this study tended to show PKP1 expressed more frequently in cytoplasm.

Cytoplasmic PKP1 expression and clinicopathological factors

Among our samples, 71.7% (71/99) showed strong cytoplasmic PKP1 staining. Cytoplasmic PKP1 expression was inversely related to tumor invasion depth (P=0.002), lymph node metastasis (P=0.003), disease stage (P=0.0004) and lymphatic invasion (P=0.001; Table 2).

Membrane PKP1 expression and clinicopathological factors

Of our samples, 45.5% (45/99) showed strong membrane staining. As with cytoplasmic expression, membrane PKP1 expression was inversely related to tumor invasion depth (P=0.00007), lymph node metastasis (P=0.001), disease stage (P=0.0001) and lymphatic invasion (P=0.001); and additionally, to venous invasion (P=0.001). These relationships tended to be stronger than those for cytoplasmic expression (Table 3).

Nuclear PKP1 expression and clinicopathological factors

Among our samples, 50.5% (50/99) showed strong nuclear PKP1 staining, which was inversely related to invasion depth (P=0.02), lymph node metastasis (P=0.004) and disease stage (P=0.006); but not to other tested factors (Table 4).

PKP1 expression and tumor recurrence

Among these 99 patients, recurrences were confirmed in 20 (20.2%). As PKP1 affects cell adhesion and migration, we investigated its effects on distant metastasis. Twelve of the 20 patients with recurrent disease had distant metastasis. Although patients with weak cytoplasmic expression tended to get metastasis, the other PKP1 staining patterns were not significantly related to distant metastasis (Table 5).

PKP1 staining patterns and patient survival

Overall survival (OS) and disease-free survival (DFS) were analyzed with respect to staining in all three cellular areas. With regard to cytoplasmic staining, 5-year OS of the strongly-staining group was significantly better (81.7%) than with the weakly-staining group (60.7%; P=0.0187; Figure 2A). However, 5-year DFS did not significantly differ between the strongly- and weakly- staining groups (P=0.18; Figure 2B).

As for membrane staining, 5-year OS did not significantly differ between the strongly-

and weakly-staining groups (84.4% vs 68.5%; *P*=0.0912; Figure 2C). However, DFS significantly differed between the groups (*P*=0.0111) Figure 2D).

Nuclear staining showed no significant differences between the two groups for both OS and DFS (Figure 2E, F).

Discussion

Several junctional proteins reportedly have important functions in carcinogenesis, tumor invasion and metastasis. Contact between epithelial cells is mediated by several types of cell–cell junctions, which comprise a complex array of transmembrane proteins and plaque proteins (8–12). Structurally, PKPs have a central core of 10 armadillo repeats flanked by N- and C-terminal domains. The PKP domains function as binding sites for various cellular proteins, including members of the cadherin family, desmoplakin, and actin and keratin filaments (6). They help link other desmosomal proteins and recruit intermediate desmosomal proteins, and thus confer stability and adhesion to cells and tissues during normal development (13–15). Previous studies have reported stronger PKP1 staining intensity in well-differentiated areas than in less differentiated areas (16,17). Compared with normal epithelium, tumor desmosomes reportedly show reduced or absent PKP expression, especially of PKP1 and PKP3 (6).

In this study, cytoplasmic, membrane and nuclear staining for PKP1 indicated that its expression generally attenuated as the tumor progressed; that is, as staining intensity decreased, the tumor invaded deeper, lymph node metastasis spread more, and tumor stage worsened; venous infiltration also seemed to worsen. Distant metastasis tended to be more common in patients with weak PKP1 staining than in those with strong staining. As PKP1 is involved in cell adhesion, these results seem to be reasonable. In some disorders, PKP1 suppression leads to uncontrollable cell adhesion, and carcinogenesis may be provoked. If PKP1 is modified during carcinogenesis, the tumor may progress because of uncontrolled adhesion and migration. As PKP1expression reflects tumor activity, it may plausibly be a predictor of EC progression and prognosis.

Of the other PKPs, elevated PKP2 has been associated with several tumor types (18,19), mainly in adenocarcinomas, but has been reported in colorectal, prostate, oropharyngeal and bladder cancers and in gliomas. PKP3 is ubiquitously expressed in all layers of the epithelium except hepatocytes, and helps regulate protein synthesis, proliferation regulation, and transcription. It is observed in various types of cancers, including colon, lung, and bladder cancers. Neither PKP3 nor PKP2 were examined in this study. We intend to investigate these two PKPs in the future.

In conclusion, PKP1 inhibits cell proliferation, colony formation, migration, and

invasion, and enhances apoptosis; its expression is inversely related to malignancy. The present study showed consistent results in terms of depth, lymph node metastasis, stage, and OS, and indicated that PKP1 may be a tumor suppressor and potential prognostic marker for EC.

Conflicts of interest and source of funding

The authors declare no conflicts of interest, and they received no financial support specifically for this study.

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Figure legends

Figure 1. Plakophilin-1 immunostaining in normal and cancerous esophageal tissues. A: Strong cytoplasmic staining in normal epithelial cells (positive control). B: Strong positive cytoplasmic staining in tumor tissue. C: Weak positive cytoplasmic staining in tumor tissue. D: Strong positive membrane staining in tumor tissue. E: Weak positive membrane staining in tumor tissue. F: Strong positive nuclear staining in tumor tissue. G: Weak positive nuclear staining in tumor tissue.

Figure 2. Kaplan–Meier survival curves for each PKP1 staining status. A: Overall survival (OS) by cytoplasmic staining status. B: Disease-free survival (DFS) by cytoplasmic staining status. C: OS by plasma membrane staining status. D: DFS by plasma membrane staining status. E: OS by nuclear staining status. F: DFS by nuclear staining status.

Table 1. Clinical characteristics of patients with esophageal cancer

Characteristics	All(n=99)
Age(mean),range	68.1±9.7
Gender	
male	86
female	13
Localization	
Upper	14
Middle	52
Lower	33
Depth of invasion	
T1a	37
T1b	26
T2	10
T3	24
T4a	1
T4b	1
Lymph node metastatis	
N0	75
N1	14
N2	5
N3	5
Stage	
0	16
ⅠA	21
ⅠB	24
ⅡA	5
ⅡB	11
ⅢA	3
ⅢB	14
ⅣA	5
Lymphatic invasion	32
Venous invasion	45
Status	
survival	69
death	30

Table 2. Relationship between cytoplasmic expression of PKP1 and clinicopathological parameters

	PKP1		*p* value
	strong	weak	
Age(mean),range	68.1±9.7	68.6±9.3	
Gender			0.509
male	63	23	
female	8	5	
Localization			0.459
Upper	12	2	
Middle	37	15	
Lower	22	11	
Depth of invasion			
T1a	33	4	0.002
T1b	20	6	
T2	4	6	
T3	12	12	
T4a	1	0	
T4b	1	0	
Lymph node metastatis			
N+	11	13	
N−	60	15	0.003
Stage			
0-1	52	9	0.0004
2-3B	17	16	
3C-4	2	3	
Lymphatic invasion			
＋	16	16	
−	55	12	0.001
Venous invasion			
＋	16	29	
−	42	12	0.18

Table 3. Relationship between PKP1 membrane expression and clinicopathological parameters

	PKP1		*p* value
	strong	weak	
Age(mean),range	68.6±9.5	68.2±9.7	
Gender			
male	42	44	
female	3	10	
Localization			
Upper	6	8	0.644
Middle	26	26	
Lower	13	20	
Depth of invasion			
T1a	28	9	0.00007
T1b	9	17	
T2	4	6	
T3	4	20	
T4a	0	1	
T4b	0	1	
Lymph node metastatis			
N+	4	34	0.001
N−	41	20	
Stage			
0-1	37	24	0.0001
2-3B	8	25	
3C-4	0	5	
Lymphatic invasion			
＋	7	25	0.001
−	38	29	
Venous invasion			
＋	12	33	0.001
−	33	21	

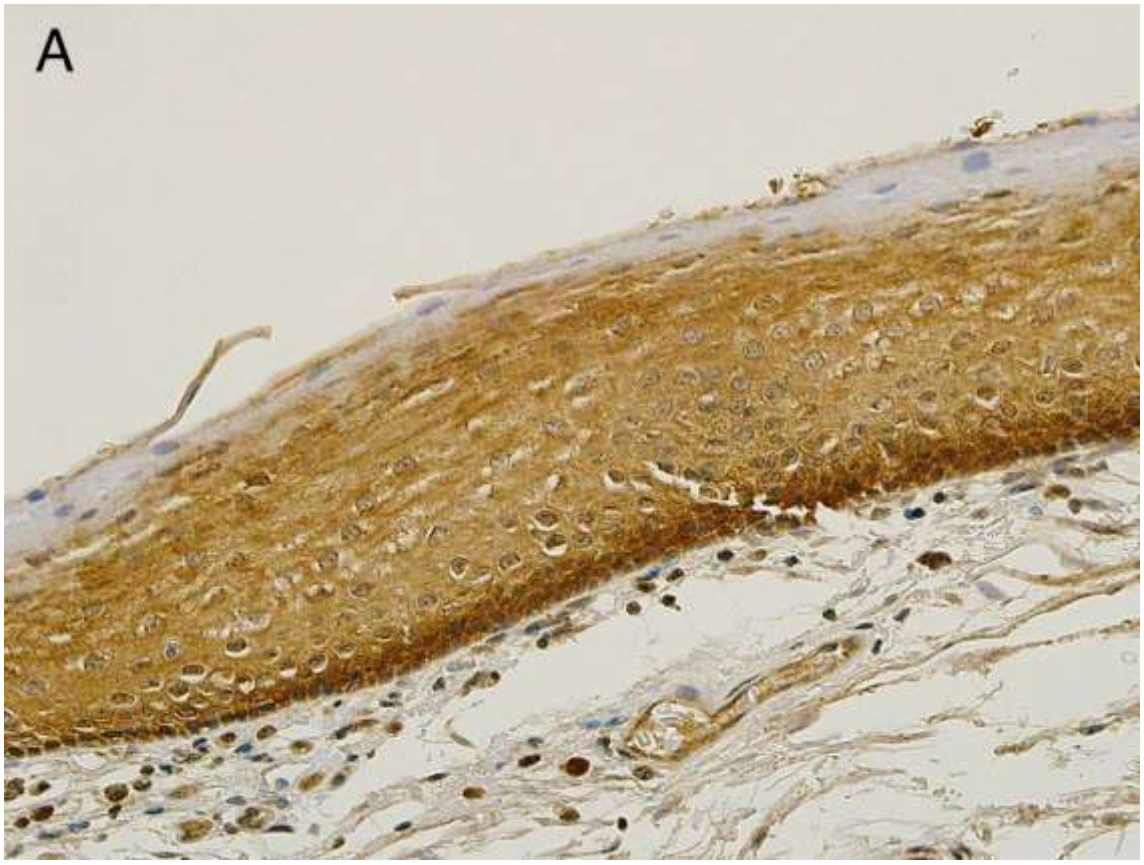
Table 4. Relationship between nuclear expression and clinicopathological parameters of PKP1

	PKP1		*p* value
	strong	weak	
Age(mean),range	67.6±9.4	68.3±9.3	
Gender			
male	43	43	
female	7	6	
Localization			
Upper	10	4	0.19
Middle	26	26	
Lower	14	19	
Depth of invasion			
T1a	23	14	0.02
T1b	15	11	
T2	6	4	
T3	6	18	
T4a	0	1	
T4b	0	1	
Lymph node metastatis			
N＋	6	18	0.004
N－	44	31	
Stage			
0-1	38	23	0.006
2-3B	10	23	
3C-4	2	3	
Lymphatic invasion			
＋	12	20	0.08
－	38	29	
Venous invasion			
＋	20	25	0.31
－	30	24	

Table 5. Recurrent cases with distant metastases

recurrent(n=20)

	Cytoplasm PKP1		*p* value	Membrane PKP1		*p* value	Nucleus PKP1		*p* value
distant metastases	strong	weak		strong	weak		strong	weak	
+	9	3	0.16	1	11	0.25	6	6	0.67
−	3	5		3	5		3	5	



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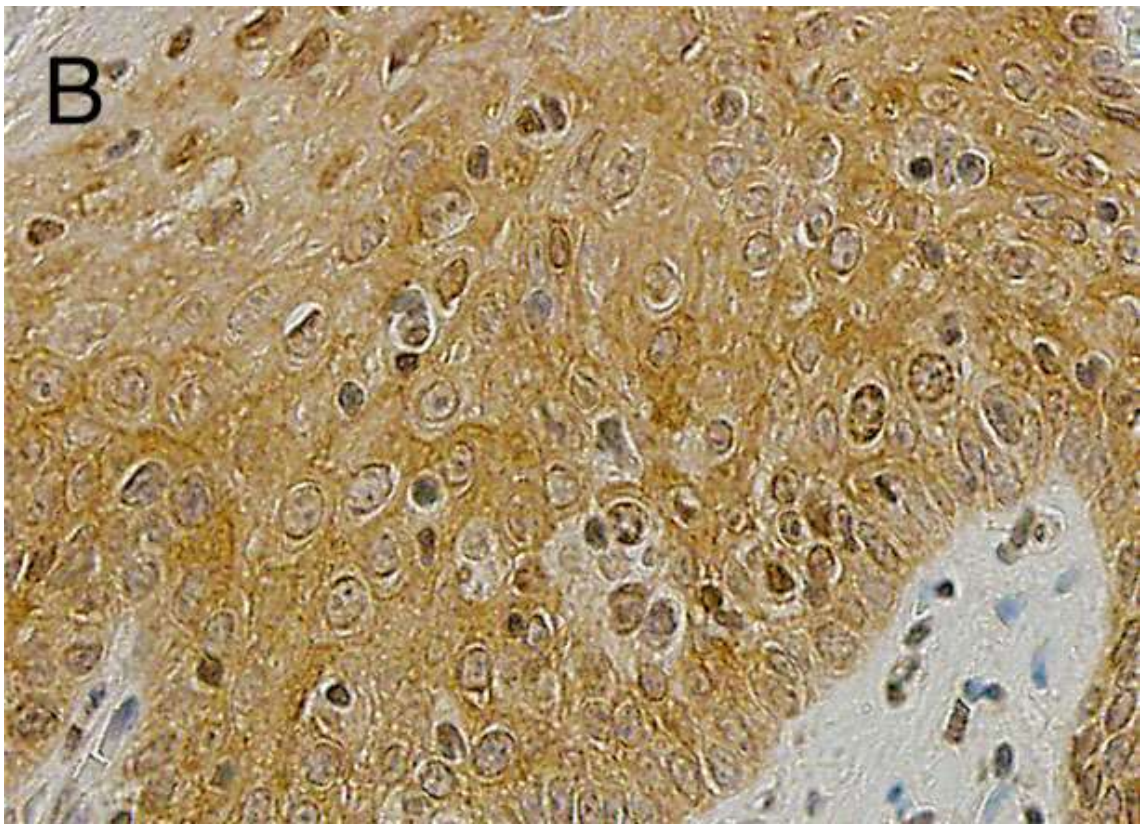

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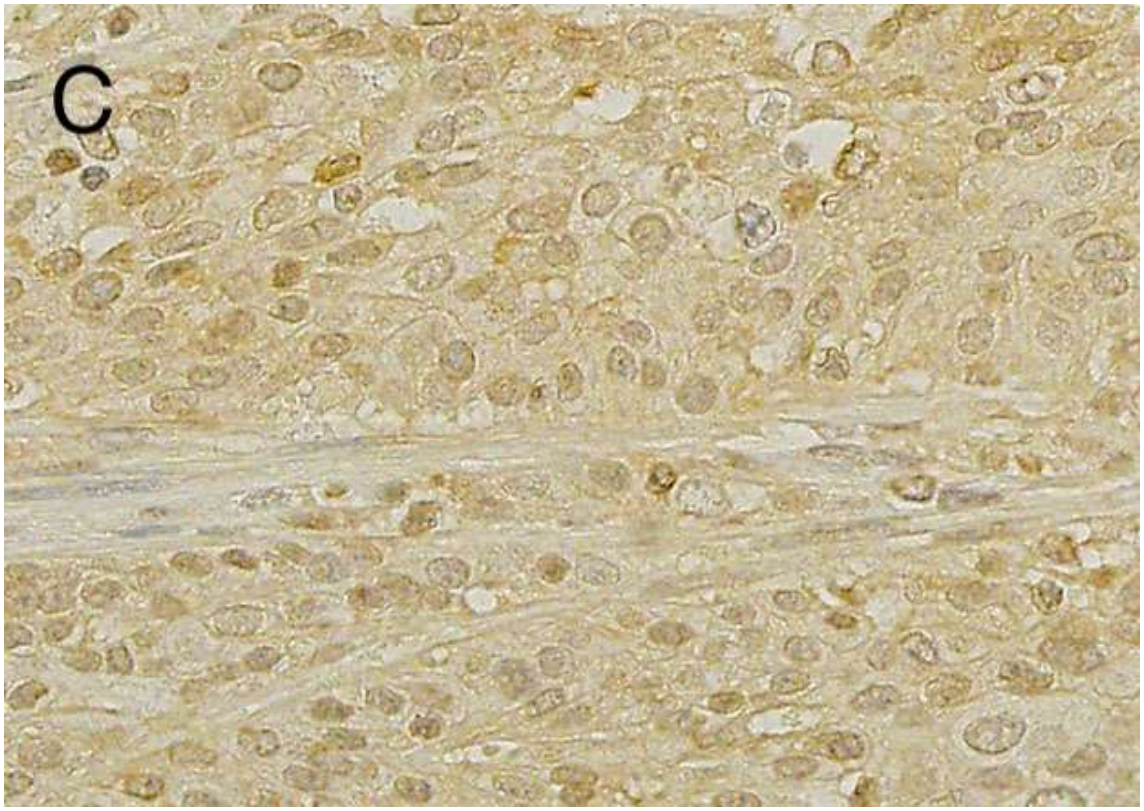

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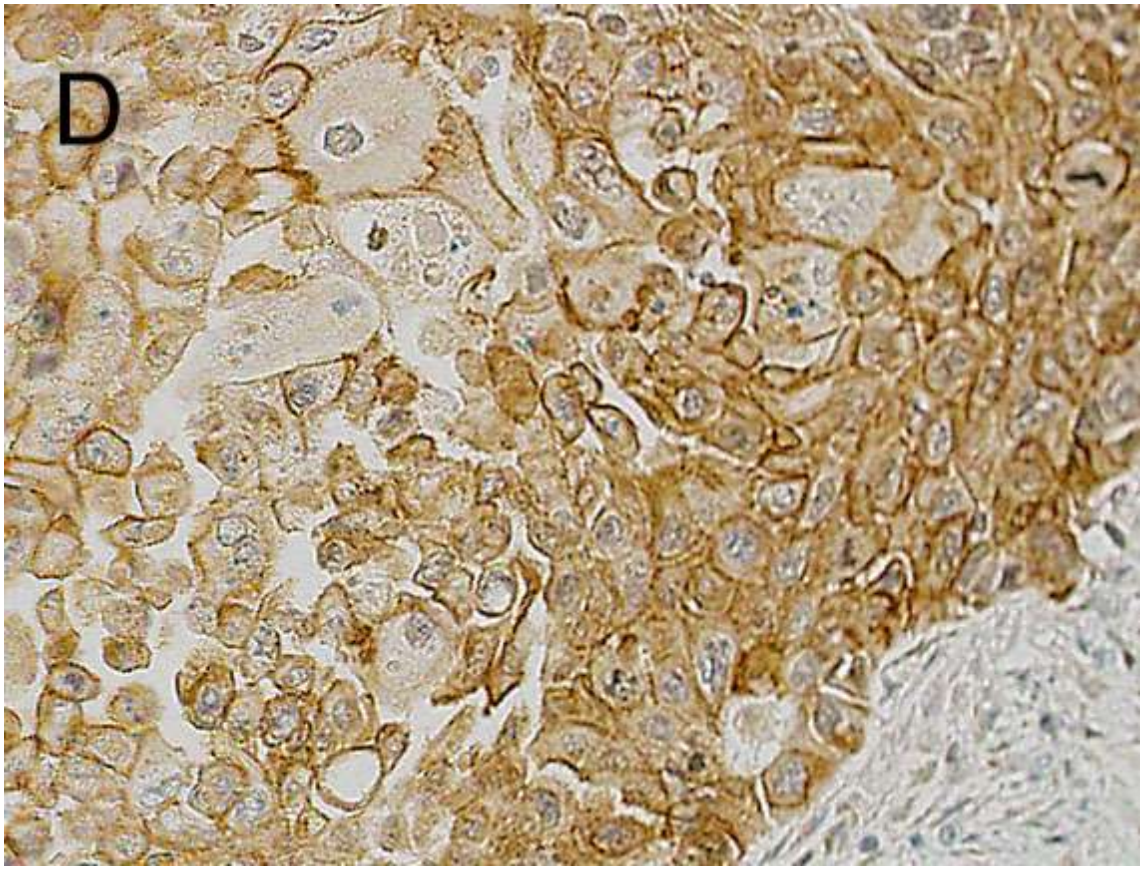
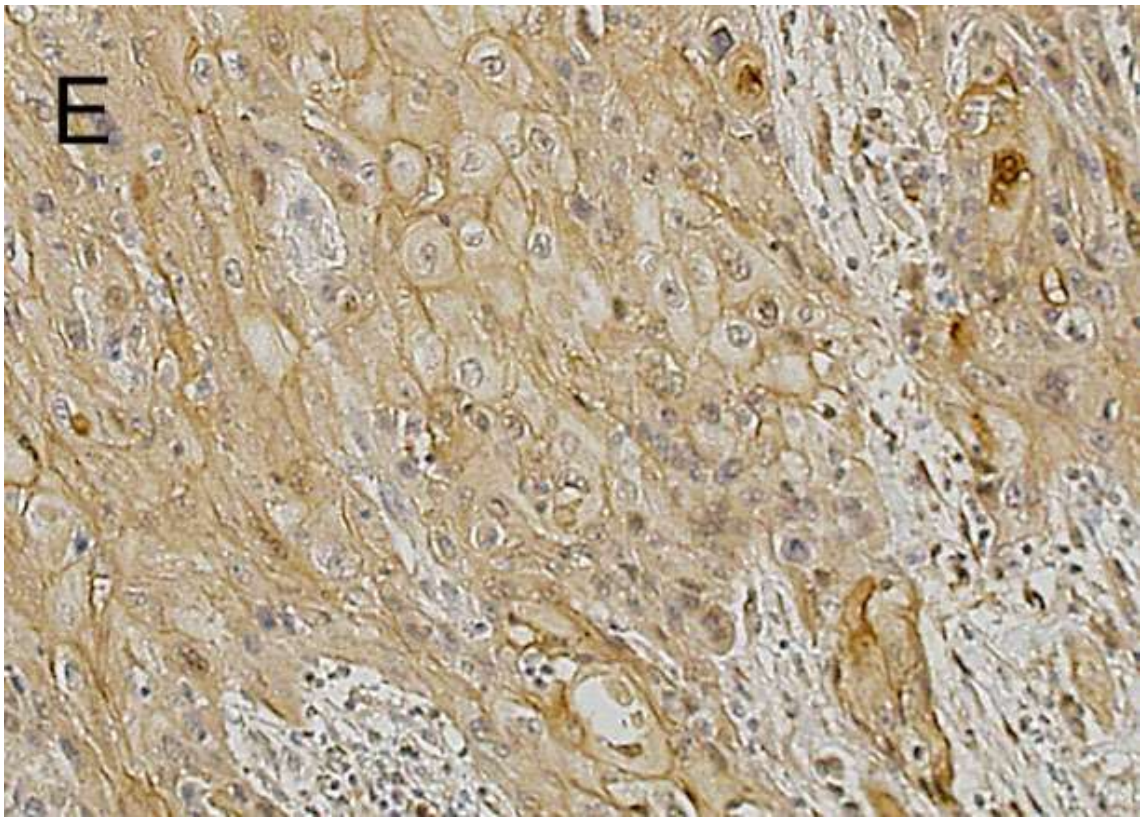


Figure1D



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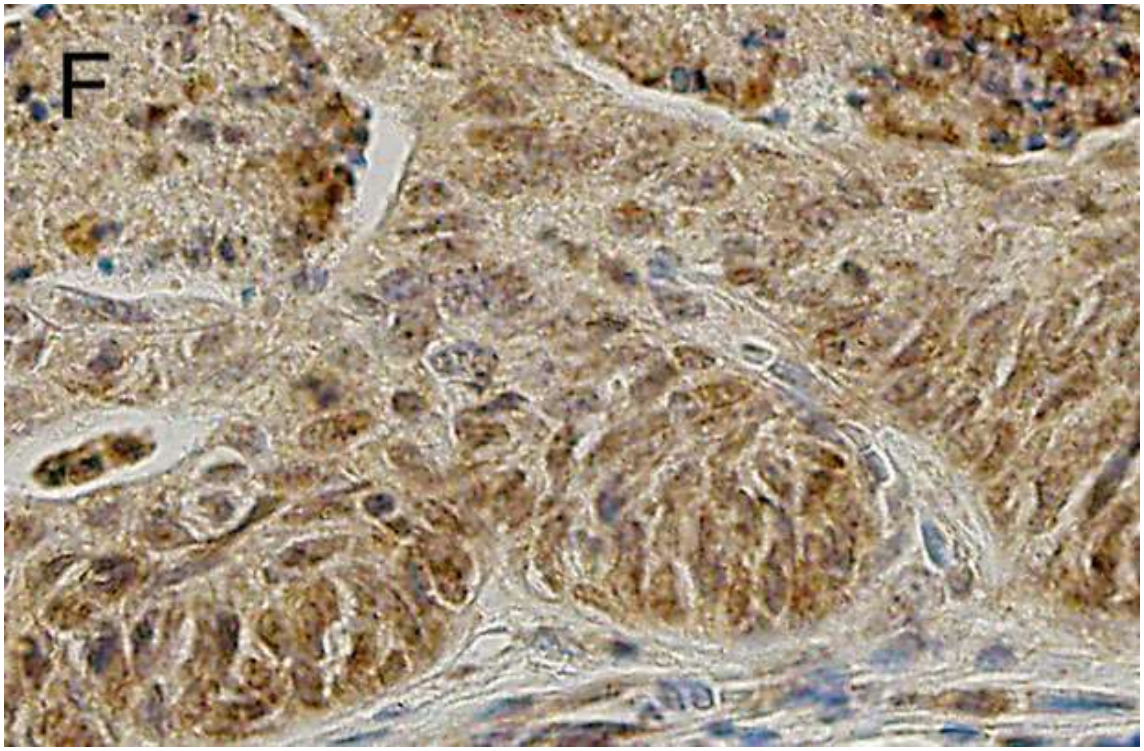

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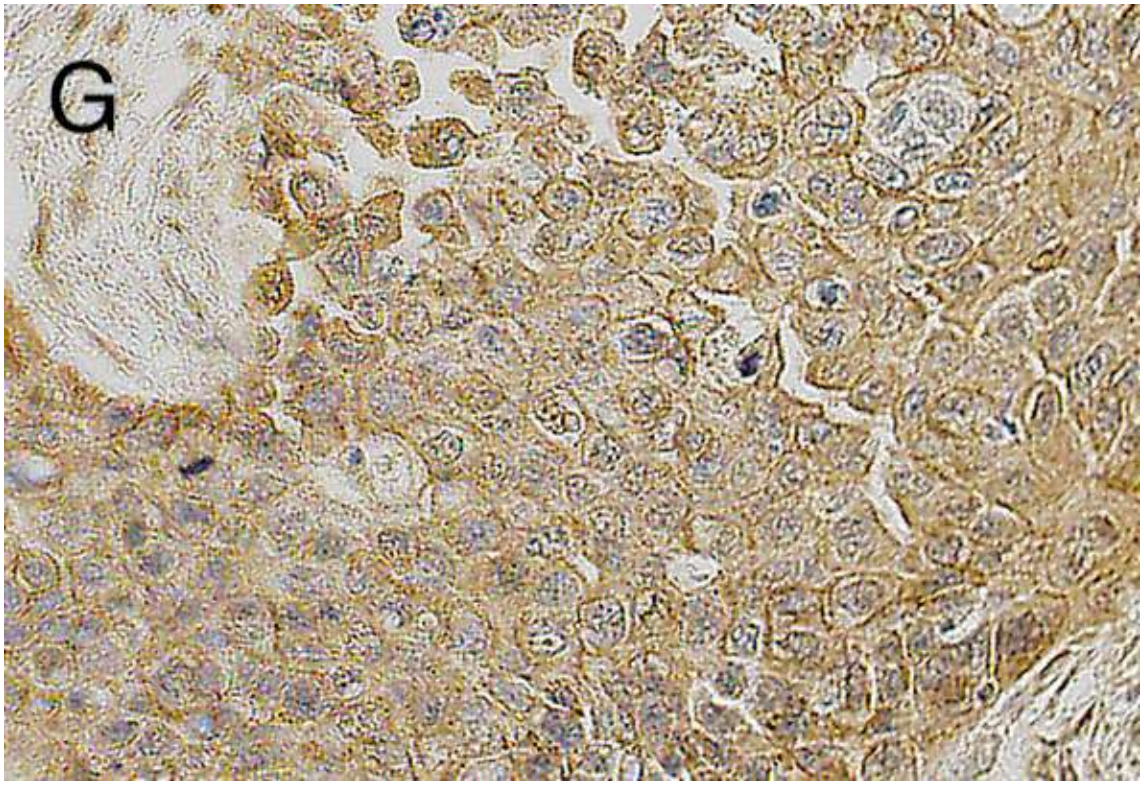

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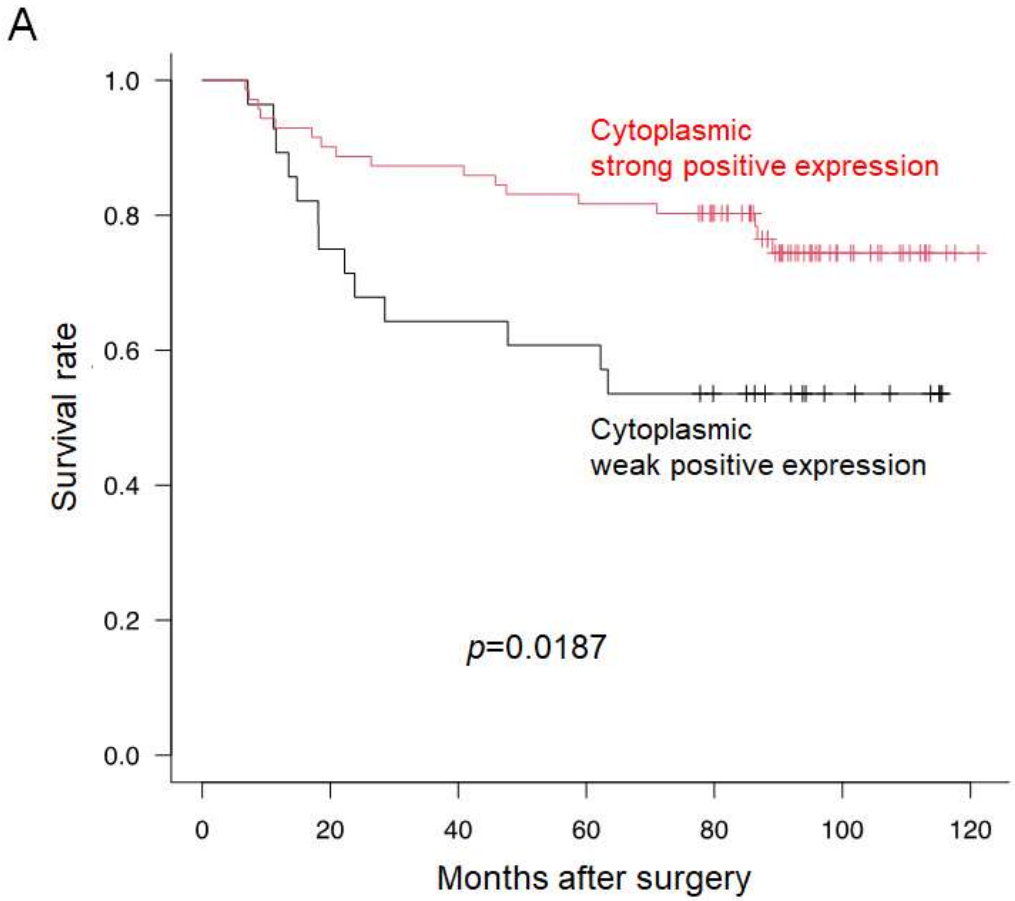
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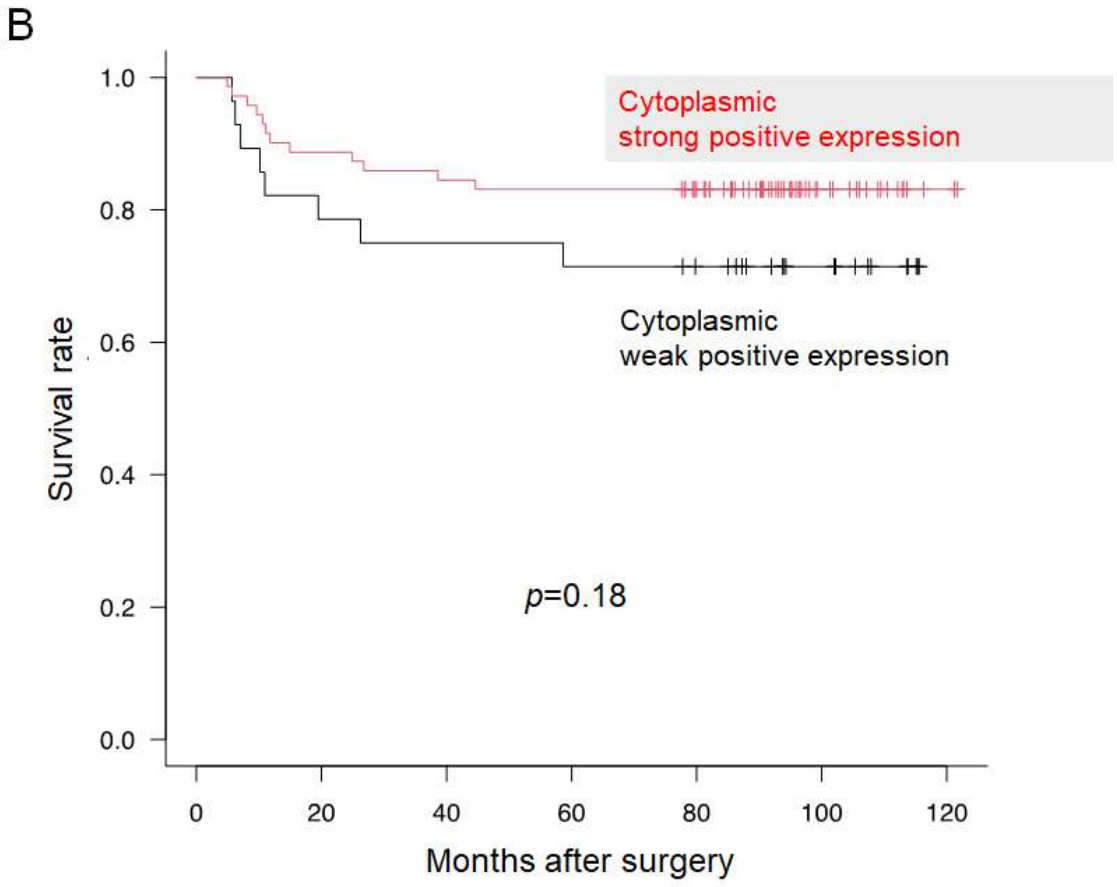
1.0
0.8
0.6
0.4
0.2
0.0
Survival rate
Cytoplasmic
strong positive expression
Cytoplasmic
weak positive expression
p=0.0187
0
20
40
60
80
100
120
Months after surgery

Figure2A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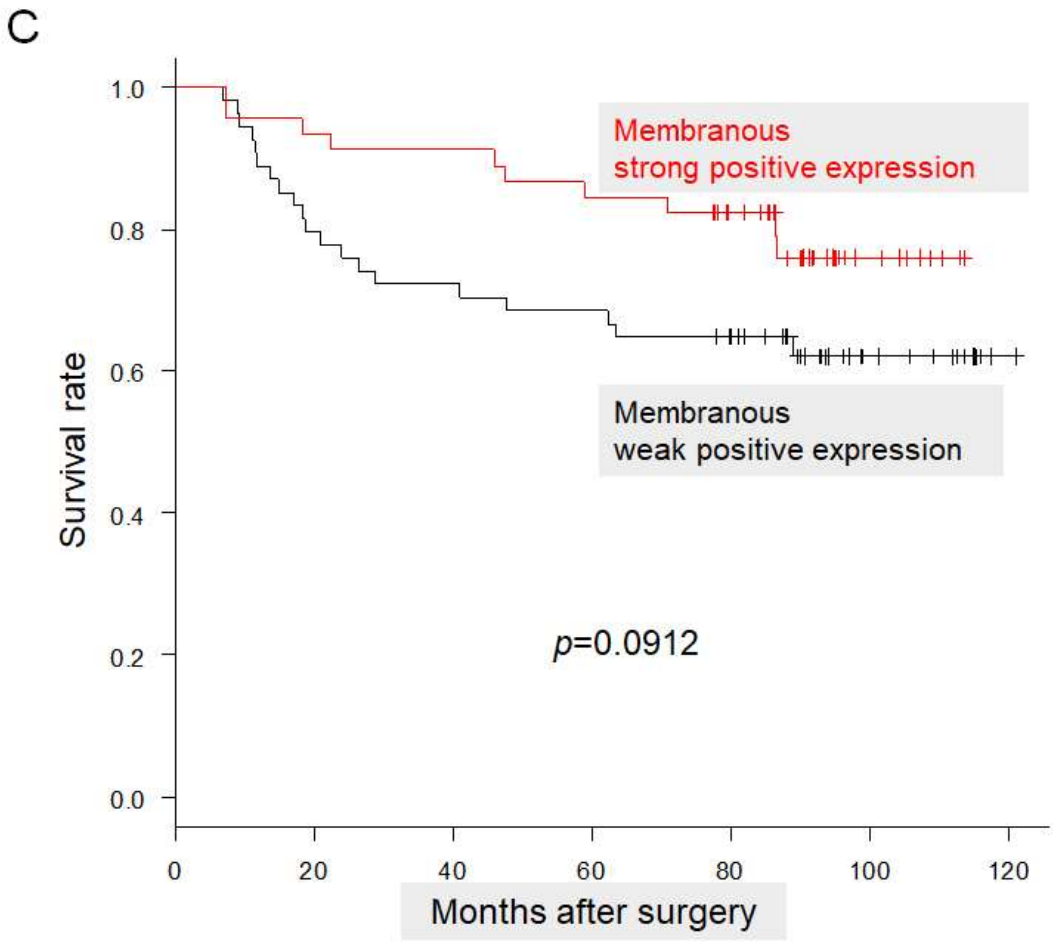
0.2
0.0
Survival rate
Cytoplasmic
strong positive expression
Cytoplasmic
weak positive expression
p=0.18
0
20
40
60
80
100
120
Months after surgery

Figure2B


C
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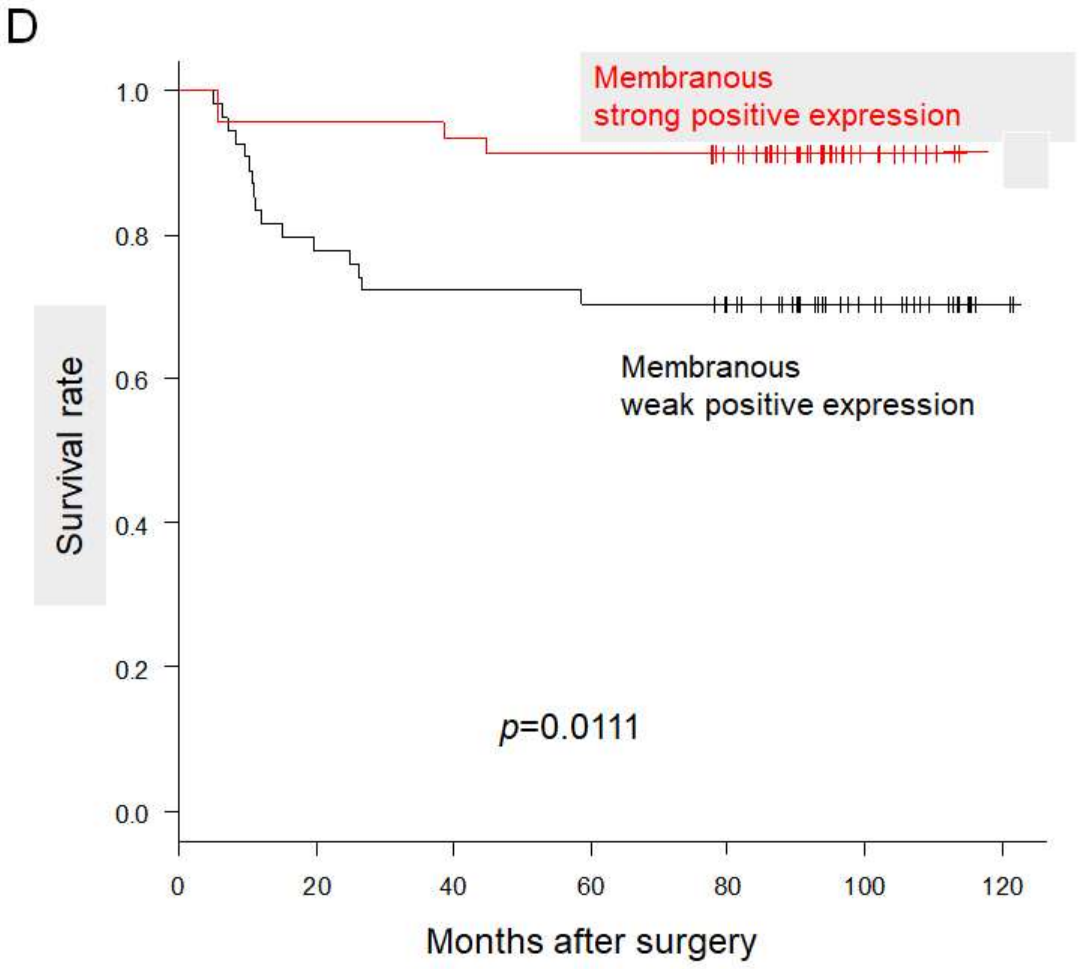
Survival rate
Membranous
strong positive expression
Membranous
weak positive expression
p=0.0912
0
20
40
60
80
100
120
Months after surgery

Figure2C


D
Membranous
strong positive expression
Membranous
weak positive expression
Survival rate
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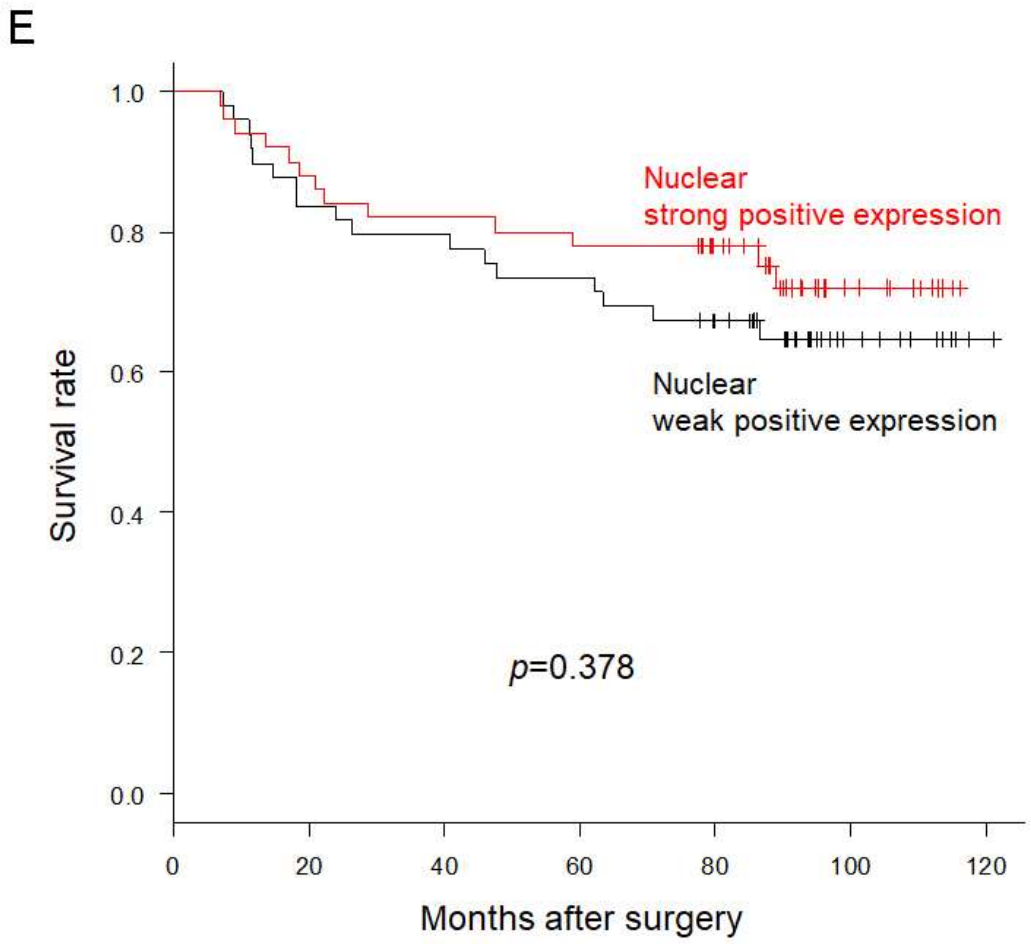
Months after surgery

Figure2D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1.0
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0.6
0.4
0.2
0.0
Survival rate
Nuclear
strong positive expression
Nuclear
weak positive expression
p=0.378
0
20
40
60
80
100
120
Months after surgery

Figure2E

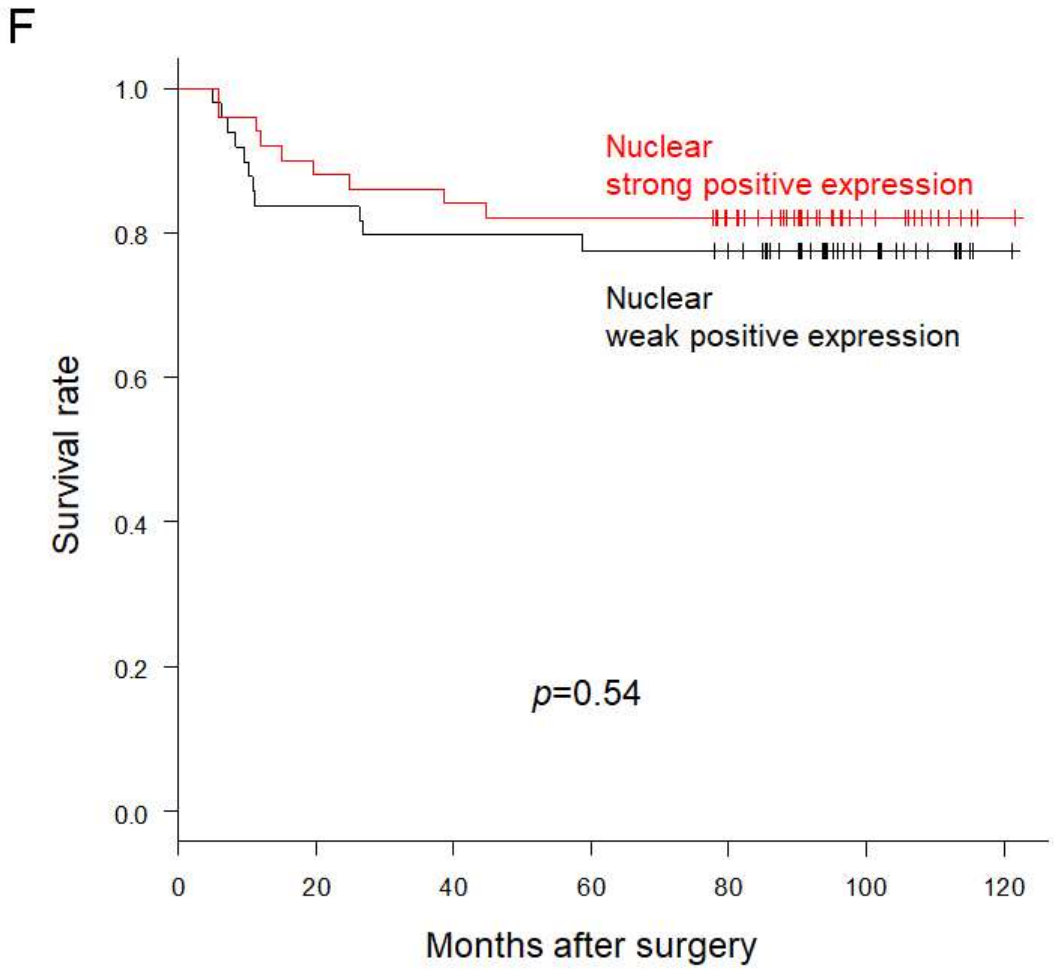
F
1.0
0.8
0.6
0.4
0.2
0.0
Survival rate
Nuclear
strong positive expression
Nuclear
weak positive expression
p=0.54
0
20
40
60
80
100
120
Months after surgery

Figure2F