Original Article

Immunohistochemical detection of a specific receptor for lipocalin2 (Solute Carrier Family 22 member 17, SLC22A17) and its prognostic significance in endometrial carcinoma

Running title: Lipocalin2 receptor in endometrial carcinoma

Tsutomu Miyamoto, M.D., Ph.D.*, Ryoichi Asaka, M.D., Akihisa Suzuki, M.D, Akiko Takatsu, M.D., Hiroyasu Kashima, M.D., Ph.D. and Tanri Shiozawa, M.D., Ph.D.

Department of Obstetrics and Gynecology, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan

***Address correspondence to**: Miyamoto T: Department of Obstetrics and Gynecology, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan

Conflict of interest

The authors declare that they have no conflict of interest.

Abstract

Background: We previously reported the overexpression of lipocalin2 (LCN2), a 25kDa secretory protein involved in iron-transportation, in endometrial carcinoma and its possible contribution to endometrial carcinogenesis. Recently, a specific receptor for LCN2, solute carrier family 22 member 17 (SLC22A17), was identified. The present study was undertaken to investigate the expression of SLC22A17 in endometrial carcinoma.

Methods: The expression of the SLC22A17 and LCN2 proteins was examined immunohistochemically using 69 cases of endometrial carcinoma and adjacent normal endometrial tissues. Immunoreactivity was evaluated according to the percentage of positive cells and described as a positivity index (PI, full score 100).

Results: The expression of SLC22A17 was negligible in normal endometria, but positive staining for SLC22A17 (PI≧1) was observed in 35 cases of endometrial carcinoma. The PI for SLC22A17 was significantly higher in cases with histological grade 3 (P<0.0005), advanced FIGO stage (*P*=0.002), deep myometrial invasion (*P*=0.029), positive lymph-vascular space invasion (*P*=0.029), positive intraperitoneal cytology (*P*=0.020) and adnexal metastasis (*P*=0.029). The expression of SLC22A17 and LCN2 was positively correlated with a significant difference (P=0.002), and the patients who overexpressed both SLC22A17 and LCN2 showed poorer survival than those without the expression of SLC22A17 or LCN2 (p=0.002). Moreover, the overexpression of both SLC22A17 and LCN2 was indicated to be an independent prognostic factor by multivariable analysis.

Conclusions: These results suggested SLC22A17, in cooperation with LCN2, to be involved in the acquisition of aggressive behavior among endometrial carcinoma cells.

Key words: endometrium, endometrial carcinoma, SLC22A17, lipocalin2, immunohistochemistry

Introduction

Endometrial carcinoma is one of the most common malignancies in the female genital tract (Ronnett et al., 2002). Its morbidity and mortality has also been increasing rapidly in Japan (Matsuda et al., 2011). Thus, further understanding of this malignancy's carcinogenetic and developmental processes and biological characteristics is important for better management of the disease. Previous studies reported the accumulation of genetic abnormalities, such as mutations of *PTEN*, *K-ras* and *p53* and microsatellite instability, to be involved in endometrial carcinogenesis (Lax, 2004), however, the molecular abnormalities of endometrial carcinoma are not well understood.

To further isolate genes affecting endometrial carcinogenesis, we previously performed laser-captured microdissection and a microarray analysis using surgically extirpated normal and neoplastic endometrial tissues. Subsequently, we identified *lipocalin2* (*LCN2*) as a gene whose expression was highly up-regulated in endometrial carcinoma as compared with normal or hyperplastic endometrial epithelium (Miyamoto et al., 2011). Lipocalin2 (LCN2), also referred to as neutrophil gelatinase-associated lipocalin (NGAL) or 24p3, is a 25kDa soluble and secretory protein. *24p3* was originally isolated from SV40-infected mouse kidney cells (Hraba-Renevey et al., 1989). Human NGAL, a homologue of the mouse 24p3, was isolated as a protein which formed a complex with a 92kDa gelatinase in neutrophils (Kjeldsen et al., 1993) and was increased in serum of patients with acute phase bacterial infections (Xu et al., 1995). LCN2 was then revealed to act in a novel iron (siderophore) transport pathway independent of transferrin (Goetz et al., 2002; Yang et al., 2002). Later studies elucidated additional functions, such as protecting MMP-9 against degradation (Yan et al., 2001) and inducing apoptosis in pro-B cells (Devireddy et al., 2001), and in

endometrial carcinoma cells (Lin et al., 2007).

We previously demonstrated that the immunohistochemical expression of LCN2 was increased in higher grade and advanced stage endometrial carcinomas, and that overexpression of the LCN2 protein was associated with shorter survival periods (Miyamoto et al., 2011). The up-regulation of LCN2 expression has also been reported in carcinomas of the lung, colon, pancreas, esophagus, mammary gland and ovary (Friedl et al., 1999; Stoesz et al., 1998; Zhang et al., 2007; Cho and Kim, 2009). In addition, our experiments in vitro strongly suggested LCN2 to influence the invasive and proliferative potential of endometrial carcinoma cells. However, the intra-cellular mechanisms which elicit the LCN2-related functions remain undetermined.

Recently, solute carrier family 22, member 17 (SLC22A17), also referred to as neutrophil gelatinase-associated lipocalin receptor (NGALR), was identified as a specific cell surface receptor for LCN2 (Devireddy et al., 2005). SLC22A17 reportedly has a transmembrane domain and facilitates the transport of LCN2 across cytoplasmic or internal membranes (Fang et al., 2007). The present study was undertaken to examine the expression and role of SLC22A17 in endometrial carcinoma in relation to the expression of LCN2.

Materials & Methods

Samples

Formalin-fixed and paraffin-embedded tissue specimens of endometrial carcinomas obtained by hysterectomy were selected from the pathology files of Shinshu University Hospital, and used for immunohistochemistry. Sixty-nine cases of endometrial adenocarcinoma were treated between 1996 and 2005 with known age (from 33 to 90

years of age, median 57.2), several clinocopathological data [myometrial invasion, lympho-vascular space invasion (LVSI), cervical invasion, lymph node metastasis, intraperitoneal cytology and adnexal metastasis] and follow-up survival data. According to the International Federation of Gynecology and Obstetrics (FIGO) classification (2008), 47 patients had stage I or II tumors, and 22 had stage III or IV tumors. The histological diagnosis was made by two pathologists in the Department of Laboratory Medicine of the hospital. Histologically, 67 patients had endometrioid adenocarcinomas and 2 had nonendometrioid carcinomas. Of the 67 endometrioid carcinomas, 43 were grade 1, 9 were grade 2, and 15 were grade 3. Of the 2 nonendometrioid carcinomas, 1 was a serous papillary adenocarcinoma, and 1 was a clear cell carcinoma. Four cases of normal endometrium (2 in a proliferative phase and 2 in a secretory phase) were observed beside the endometrial carcinoma tissues. Each tissue sample was used with the approval of the Ethics Committee of Shinshu University, after obtaining written consent from the patients.

Immunostaining

Indirect immunohistochemical staining was performed using a rabbit-polyclonal anti-SLC22A17 antibody (ABbiotec, San Diego, CA), rat-monoclonal anti-human lipocalin2 antibody (R & D systems, Minneapolis, MN), and Histofine MAX-PO detector kit (Nichirei, Tokyo, Japan) with microwave pretreatment as described previously (Shiozawa et al., 2001). Tissue sections of brain for the anti-SLC22A17 antibody, and of breast carcinoma for the anti-LCN2 antibody, were used as a positive control.

Evaluation of immunoreactivity and statistical analysis

Immunoreactivity was evaluated according to the percentage of positive cells among 500 cells in 5 high power fields by two independent reviewers (T.M. and T.S.), and these results were described as a positivity index (PI), with a maximal score of 100. A case with PI≧1 was defined as positive, and that with PI≧10 as strongly positive according to our previous study (Miyamoto et al., 2011). The significance of differences in the PI among the histological grades was examined using the Kruskal-Wallis rank test and Sheffe's test. Immunoreactivity of the non-endometrioid subtypes was evaluated the same as grade 3 endometrioid carcinomas. The significance of differences in the PI of FIGO stage or other clinicopathological parameters was examined using the Mann-Whitney U test. A P value of less than 0.05 was considered significant. Cumulative survival was also analyzed using the Kaplan-Meier method. The log-rank test was used to evaluate the significance of SLC22A17 and LCN2 for survival. In addition, prognostic values of clinicopathological variables and immunoreactivity of SLC22A17/LCN2 were evaluated by Cox regression analysis. These analyses were conducted using the SPSS Statistics system (SPSS Inc., Chicago, IL).

Results

The expression of SLC22A17 in endometrial carcinoma and adjacent normal endometrium

Immunoreactivity for the SLC22A17 protein was observed in the cytoplasm (Fig 1). The expression of SLC22A17 was not observed in normal endometrial gland (2 secretory phase cases and 2 proliferative phase cases) (Fig 1a, b). Staining for SLC22A17 was observed in 35 cases (50.7%), of which 12 (17.4%) showed strong

staining (Fig 1c-f).

The PIs of SLC22A17 according to clinicopathological parameters were summarized in Figure 2. The PI of SLC22A17 in grade 1, grade 2, and grade 3 tumors was 2.1 ± 3.4, 4.4 ± 5.6, and 9.1 ± 9.5, respectively. The PI of grade 3 tumors was significantly higher than that of grade 1 tumors (P<0.0005). The PI of stage III and IV tumors (8.2 ± 8.9) was significantly higher than that of stage I and II tumors (2.6 ± 4.4) (P=0.002). In addition, the PI of SLC22A17 was significantly higher in cases with deep (≧1/2 of myometrium) myometrial invasion (P=0.029), positive LVSI (P=0.029), positive intraperitoneal cytology (P=0.020) and adnexal metastasis (P=0.029). In contrast, the significant difference of the PI of SLC22A17 was not observed as to lymph node metastasis and cervical invasion.

The PI of LCN2 in grade 1, grade 2, and grade 3 tumors was 5.8 ± 8.2, 29.6 ± 33.8, and 15.4 ± 22.0, respectively. The PI of grade 2 tumors was significantly higher than that of grade 1 tumors (p = 0.001). The PI of stage III and IV tumors (16.6 ± 21.7) was significantly higher than that of stage I and II tumors (8.6 ± 16.6) (p=0.040).

Correlation of the expression of SLC22A17 and LCN2

The PI of SLC22A17 was significantly correlated with that of LCN2 (P=0.002) (Fig 3). Topologically, SLC22A17 over-expression was frequently observed in areas of lympho-vascular space invasion (Fig 4a) and deep myometrial invasion (Fig 4b). This expression pattern was similar to LCN2 expression pattern (Fig 4d, e). The co-expression of LCN2 and SLC22A17 was also often observed at the apex of papillary structures (Fig 4c, f).

The expression of SLC22A17 protein and the survival of endometrial carcinoma patients

Regarding the prognostic value of SLC22A17, endometrial carcinoma patients with strong SLC22A17 expression (12/69) had significantly shorter survival periods (P=0.003, Fig 5a). Patients with strong LCN2 expression (21/69) also had a significantly shorter survival (P=0.043). We then compared the survival of the four subgroups according the results of immunostaining for SLC22A17 and LCN2 (strongly positive and negative). Cases with strongly positive staining of both SLC22A17 and LCN2 (7/69 cases) had significantly shorter survival periods than did the other subgroups (P = 0.002, Fig 5b). In addition, we calculated the prognostic value of clinicopathological parameters and immunoreactivity of SLC22A17/LCN2 using Cox regression analysis (Table 1). The univariable analysis revealed that the prognosis was significantly poorer in patients with strongly positive staining of both SLC22A17 and LCN2 (P < 0.0005), higher histological grade (grade 2 and 3) (P = 0.012), advanced FIGO stage (III and IV) (P = 0.001), deep myometrial invasion (P = 0.003), positive intraperitoneal cytology (P = 0.002) and adnexal metastasis (P < 0.0005). Among those factors, multivariable analysis revealed the strongly positive staining of both SLC22A17 and LCN2 to be an independent prognostic factor for poorer survival (P = 0.021).

Discussion

SLC22A17 was identified as a specific receptor for LCN2 (Devireddy et al., 2005). According to a database search (http://www.ncbi.nlm.nih.gov/gene/51310), SLC22A17 is part of the major facilitator superfamily (MFS). MFS proteins have a transmembrane domain and facilitate the transport across cytoplasmic or internal membranes of a

variety of substrates including ions, sugar phosphates, drugs, neurotransmitters, nucleosides, amino acids, and peptides. It should be noted that megalin was also identified as a receptor for LCN2 (Hvidberg et al., 2005). However, megalin binds numerous structurally unrelated ligands such as lipoprotein lipase, the antifibrinolytic polypeptide, aprotinin, apolipoprotein J/clusterin, vitamin D3 and lactoferrine as well as LCN2 (Moestrup et al., 1995; Kounnas et al., 1995; Nykjaer et al., 1999; Nagai et al., 2003), thus we focused on the expression of SLC22A17.

The present study demonstrated that SLC22A17 was not expressed in normal endometrial glands, but was strongly expressed in higher grade and advanced stage endometrial carcinomas. In addition, patients with SLC22A17 overexpression showed shorter survival periods. These results suggest that the LCN2 receptor contributes to the genesis and development of endometrial carcinoma. The expression pattern and prognostic value of LCN2 in the present study were similar to those observed in our previous study (Miyamoto et al., 2011). Interestingly, co-expression of SLC22A17 and LCN2 was frequently observed, and patients with staining for both SLC22A17 and LCN2 showed poorer survival. In addition, multivariable analysis indicated that the strongly positive staining of both SLC22A17 and LCN2 was an independent prognostic factor. These results suggest the expression of SLC22A17, in cooperation with LCN2, to be involved in the acquisition of aggressive behavior among endometrial carcinoma cells. The expression of LCN2 and SLC22A17 was shown to be correlated with poor patient survival in tumors like gliomas and esophageal squamous cell carcinomas (Liu et al., 2010; Du et al., 2011), however, this is the first report of the expression of SLC22A17 in endometrial carcinomas.

Our data demonstrated that the PI of SLC22A17 was significantly higher in

cases with deep myometrial invasion, and LVSI. And co-expression of SLC22A17 and LCN2 was frequently observed in areas of LVSI and deep myometrial invasion. These findings suggested that the SLC22A17 and LCN2 may be involved in LVSI and myometrial invasion. In addition, co-expression of SLC22A17 and LCN2 was also often observed at the apex of papillary structures. We speculate that SLC22A17 and LCN2 might be involved in detachment of cells from the apex of papillary structures. Indeed, the PI of SLC22A17 was significantly higher in cases with positive intraperitoneal cytology and adnexal metastasis. Thus, the up-regulation of SLC22A17 and LCN2 expression in endometrial carcinomas observed in the present study primarily suggests a positive role for the SLC22A17/ LCN2 system in carcinogenesis and tumor development. This interpretation is supported by our previous experiments, in which the forced expression of LCN2 in endometrial carcinoma cells increased their proliferative and invasive potential (Miyamoto et al., 2011). Similar results were reported in breast carcinoma cells; overexpressed LCN2 significantly increased cell motility and invasiveness, and induced epithelial to mesenchymal transition (Yang et al., 2009). However, an increase in LCN2 reportedly suppressed invasion and liver metastasis in colon cancer cells (Lee et al., 2006). The reason for this functional diversity is not clear. Lin and colleagues reported that LCN2 treatment induced cell apoptosis up to 48 hours in endometrial carcinoma RL95-2 cells. However, LCN2 simultaneously induced IL-8 expression which enhanced cell survival and migration after 48 hours (Lin et al., 2011).

To further understand the dualistic function of LCN2, elucidating the effects of micro-environmental iron concentrations may be important. LCN2 and SLC22A17 are known to cooperate for iron transportation. Devireddy et al. (2005) demonstrated that

LCN2 and SLC22A17 controlled the intracellular iron concentration and the depletion of intracellular iron induced apoptosis in SLC22A17-transfected HeLa cells. Holo-24p3 (iron-loaded LCN2) binds to cell surface SLC22A17, and transports iron into the cytoplasm by endocytosis. As a result, holo-24p3 increases the intracellular iron concentration and induces the iron-responsive gene expression of proteins such as ferritin. Conversely, apo-24p3 (iron-free LCN2) binds to SLC22A17 and is transported inside the cell by endocytosis. Apo-LCN2 then binds intracellular iron and is transported outside the cell by exocytosis. Consequently, apo-24p3 lowers the intracellular iron concentration, and induces production of a pro-apoptotic protein, Bim. These reports suggest that iron concentrations affect the biological role of LCN2/SLC22A17, a rationale supported by the finding that LCN2 increased cell motility and invasion through an iron-dependent mechanism (Hu et al., 2009). Although the extra- or intracellular iron concentration was not examined in the present study, the up-regulated SLC22A17 and LCN2 expression in endometrial carcinoma may be involved in iron transportation, and the altered iron concentration in turn may regulate the function of LCN2/SLC22A17. Further studies are needed to clarify the relation between LCN2/SLC22A17 and iron concentrations in endometrial carcinoma.

Regarding the molecular mechanisms of SLC22A17's up-regulation, Cui et al. described that SLC22A17 overexpression was induced by hypomethylation of the NGALR promoter (Cui et al., 2008). Sheng et al reported that SLC22A17 expression was regulated by Runx transcription factors; Runx3 activated and Runx1 repressed the expression (Sheng et al., 2009). Despite these reports, the mechanisms responsible for the increased expression of SLC22A17, as well as the co-expression of SLC22A17 and LCN2, in endometrial carcinoma remain to be elucidated.

In conclusion, we demonstrated that an increase in the SLC22A17 protein was associated with higher grade, more advanced tumors and shorter survival periods in endometrial carcinoma patients. Furthermore, SLC22A17 might cooperate with LCN2 and be involved in the progression of the disease. Although more studies are needed to clarify the molecular mechanisms of the relation between the LCN2-SLA22A17 pathway and disease progression, the results of the present study indicate this pathway to be a potential target for the treatment of endometrial carcinoma.

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Figure legends

Fig 1

Results of immunohistochemical staining for SLC22A17. Photomicrographs of immunostaining for lipocalin2 in normal endometrium in the proliferative phase **(a)**, normal endometrium in the secretory phase **(b)**, grade 2 endometrial carcinoma **(c)**, grade 3 endometrial carcinoma with deep myometrial invasion **(d)**, grade 3 endometrial carcinoma **(e)**, and grade3 endometrial carcinoma with invasion of the lympho-vascular space **(f)**

Fig 2

Results of immunohistochemical staining for SLC2A17 in relation to clinicopathological parameters.

#: significantly different from grade 1 (P<0.0005), ＊: significantly different ($p < 0.05$).

Abbreviation: MI, myometrial invasion; LVSI, lympho-vascular space invasion; CI, cervical invasion; LN, lymph node metastasis; IPC, intraperitoneal cytology; Ad, adnexal metastasis

Fig 3

Scatter graph of the PIs of LCN2 and SLC22A17.

Fig 4

Results of immunohistochemical staining for SLC22A17 **(a-c)** and LCN2 **(d-f)** in endometrial carcinoma. Co-expression of SLC22A17 and LCN2 was observed at sites of lympho-vascular space invasion **(a, d)** and deep myometrial invasion **(d, e),** and the

top of papillary structures **(c, f)**.

Fig 5

Cumulative survival of the patients with endometrial carcinoma according to the PI for immunohistochemical staining. **(a)** Immunohistochemical staining for SLC22A17. Patients with PI≧10 showed a significantly shorter survival. **(b)** Immunohistochemical staining for LCN2 and SLC22A17. The strongly positive cases with PI ≧ 10 are showed as (+), and the cases with PI < 10, as (-). The patients with LCN2 (+) / SLC22A17 (+) (thick straight line) showed significantly shorter survival periods than those with LCN2 (-) / SLC22A17 (-) (thin straight line), with LCN2 (-) / SLC22A17 (+) (thin dashed line), or with LCN2 (+) / SLC22A17 (-) (thick dashed line).

Table 1. Prognostic value of clinicopathological variables and immunoreactivity of SLC22A17/LCN2 in patients with endometrial carcinoma (Cox regression analysis)

	Univariable analysis		Multivariable analysis	
Prognostic factor	*P* value	HR (95% CI)	*P* value	HR (95% CI)
SLC22A17(2+) and LCN2 (2+)	＜0.0005	15.652 (4.104 - 59.692)	0.021	5.595 (1.291 - 24.241)
Histological grade (2 + 3)	0.012	5.567 (1.465 - 21.155)	0.37	2.101 (0.415 - 10.648)
FIGO stage (III + IV)	0.001	11.026 (2.794 - 43.518)	0.527	1.983 (0.238 - 16.558)
Deep myometrial invasion (≧1/2)	0.003	10.062 (2.139 - 47.345)	0.401	2.435 (0.306 - 19.384)
Cervical invasion	0.086	3.161 (0.848 - 11.785)		
Lymph-vascular space invasion	0.219	2.110 (0.641- 6.942)		
Lymph node metastasis	0.265	2.676 (0.473 - 15.134)		
Positive intraperitoneal cytology	0.002	6.517 (1.965 - 21.615)	0.765	1.318 (0.217 - 8.014)
Adnexal metastasis	＜0.0005	9.167 (2.758 - 30.467)	0.466	2.189 (0.266 - 18.022)

Note. P ＜ 0.05 was considered significant

Abbreviation: HR (hazard ratio), CI (confidence interval), FIGO (International Federation of Gynecology and Obstetrics), 2+ (strongly positive)

Figure 1

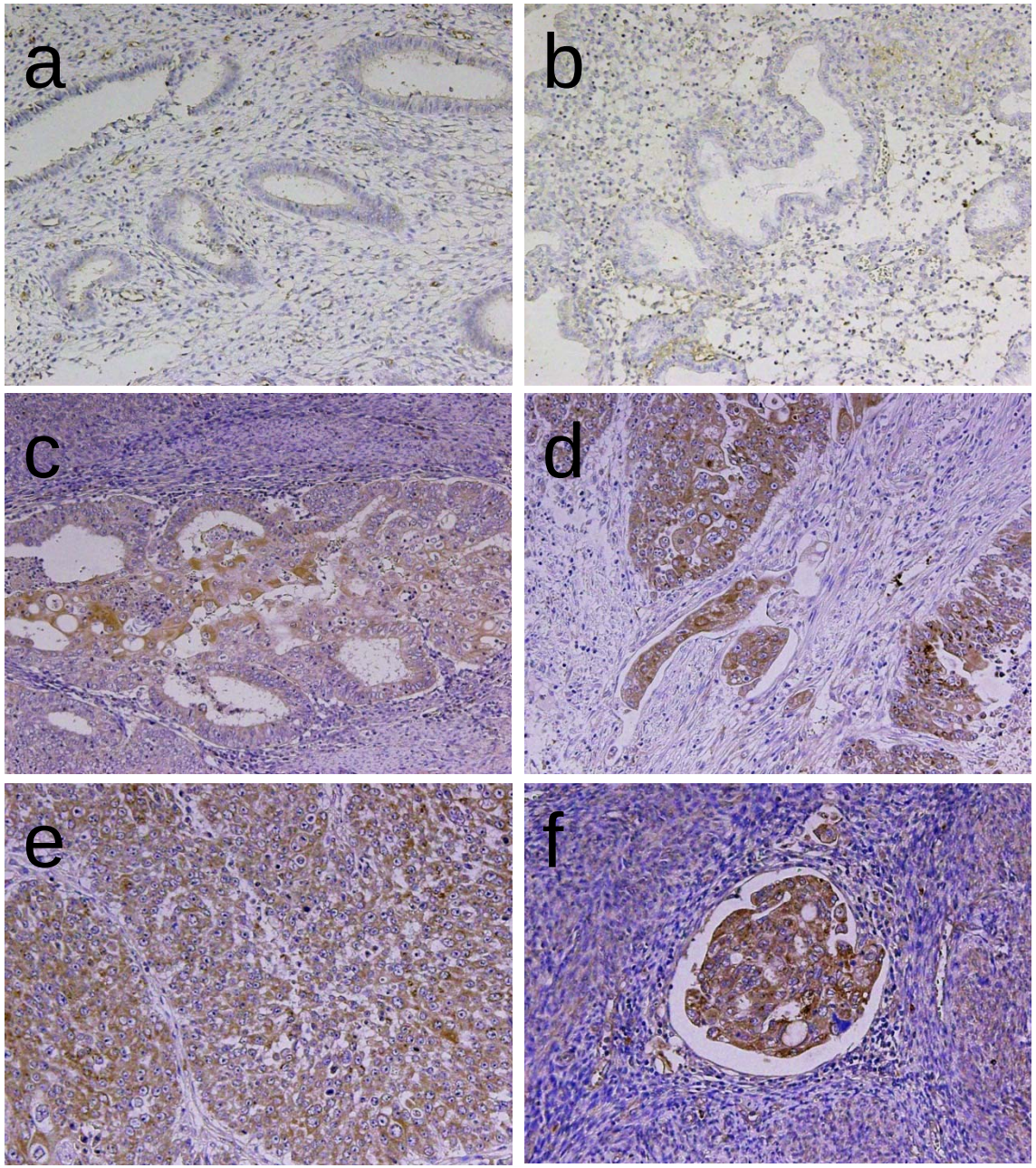

Figure 2

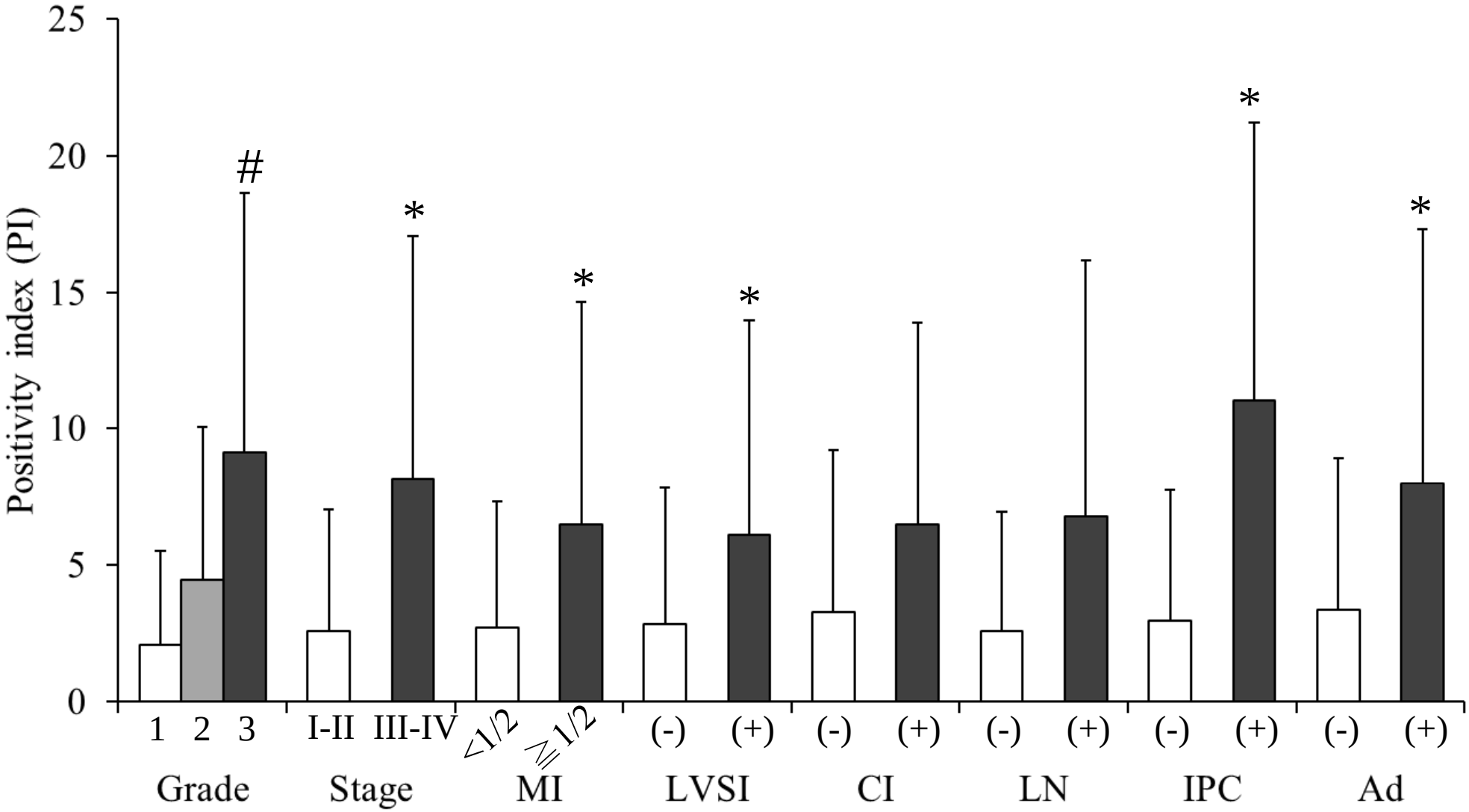
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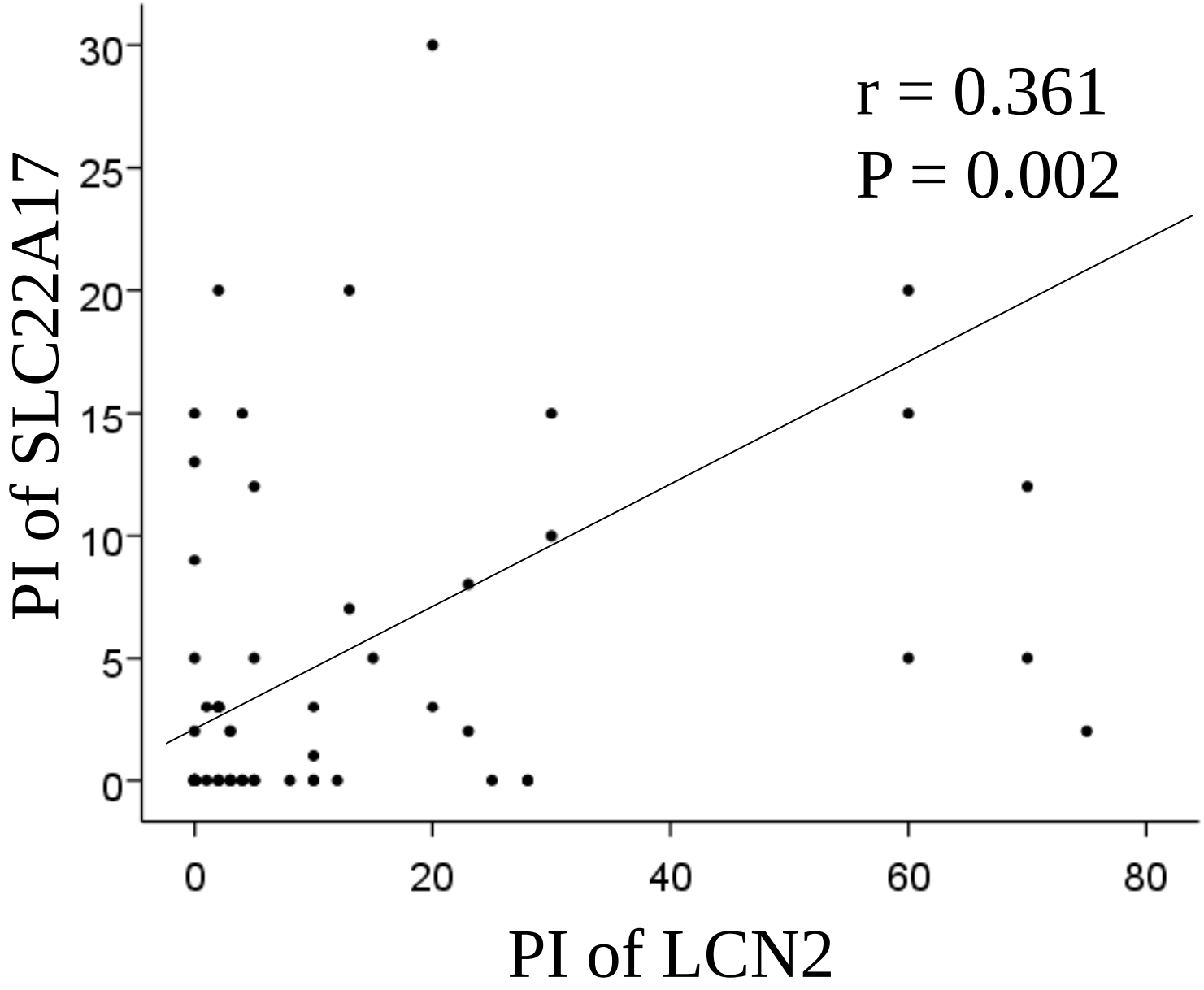
Figure 3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0
20
40
60
80

Figure 4

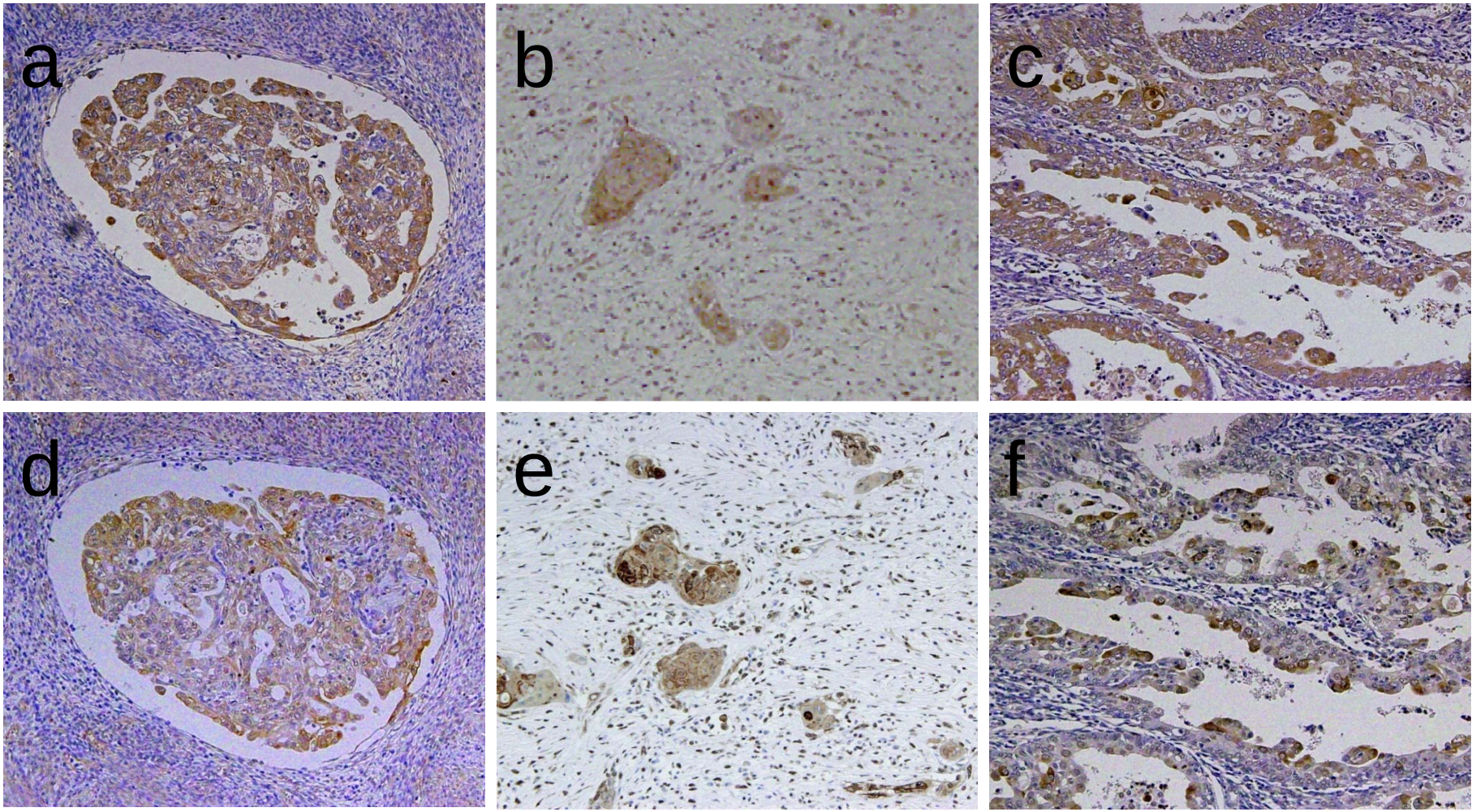
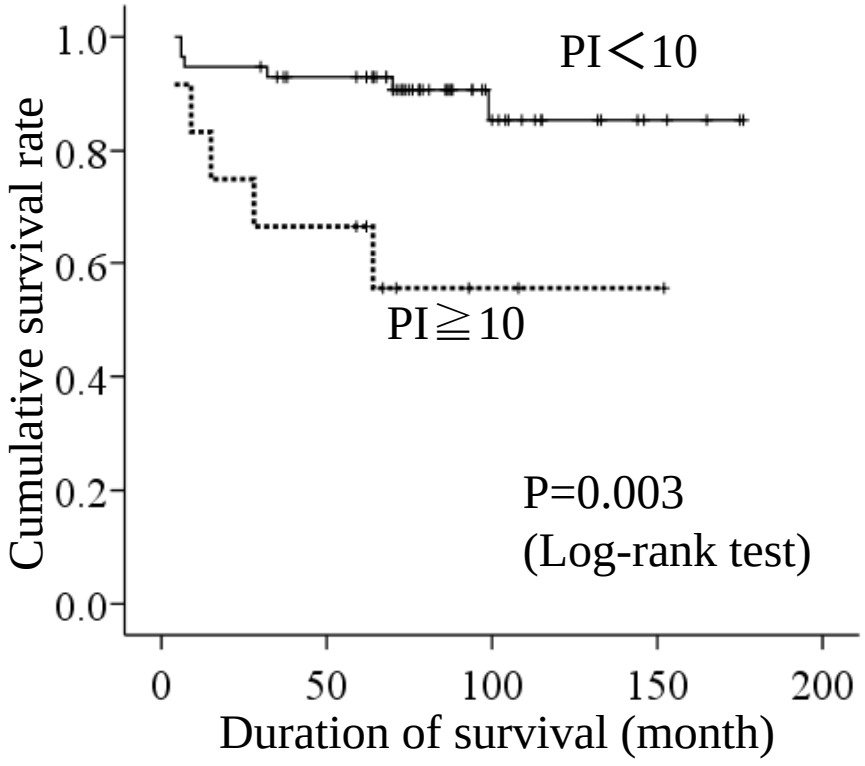
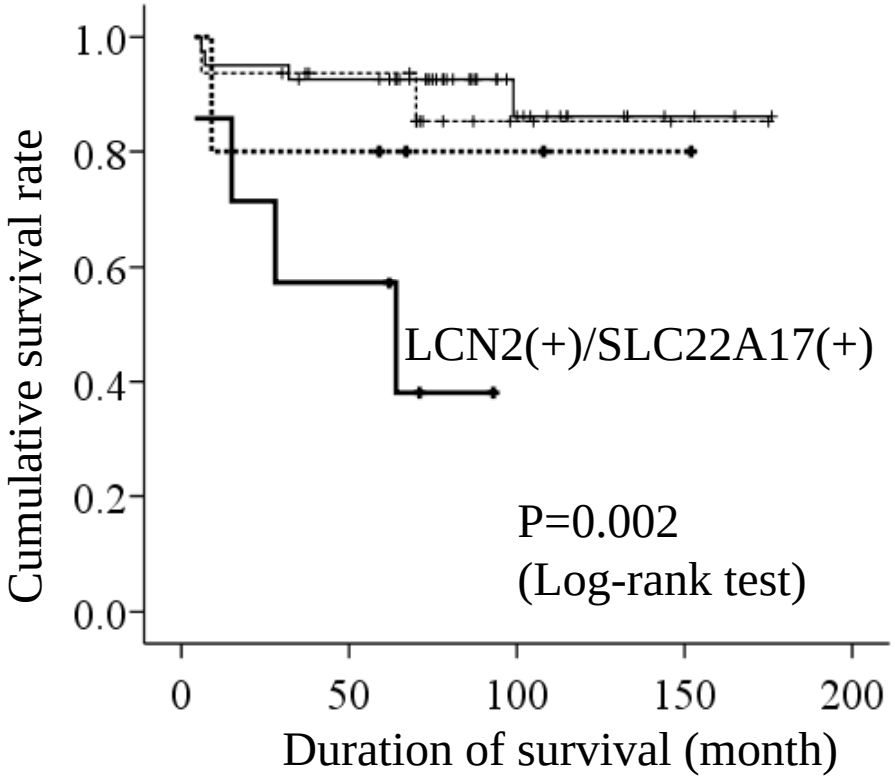


Figure 5

a


1.0
0.8
0.6
0.4
0.2
0.0
Cumulative survival rate
PI＜10
PI≧10
P=0.003
(Log-rank test)
0
50
100
150
200
Duration of survival (month)

b


1.0
0.8
0.6
0.4
0.2
0.0
Cumulative survival rate
LCN2(+)/SLC22A17(+)
P=0.002
(Log-rank test)
0
50
100
150
200
Duration of survival (month)