Elevated expression of G protein-coupled receptor 30 (GPR30) is associated with poor prognosis in patients with uterine cervical adenocarcinoma

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Summary. Uterine cervical adenocarcinoma has a worse prognosis than that of squamous cell carcinoma and useful diagnostic and prognostic markers are needed. Estrogen is one of the key regulators of several cancers, however, the estrogen signaling has not been focused on in cervical adenocarcinoma. Here, we shows expression profile of classical estrogen receptor (ER) and a novel membrane type estrogen receptor, G protein-coupled receptor 30 (GPR30), in surgical specimens (n=53). GPR30 was strongly expressed on the cell membrane and in the cytoplasm in adenocarcinoma *in situ* (AIS) and adenocarcinoma, and its expression was especially strong at the invasion front in most of the cases of GPR30-positive adenocarcinoma. Nuclear staining of ER was strong in non-neoplastic glands, whereas it was almost absent in most of the AIS and adenocarcinoma cases. There was a weak but statistically significant negative correlation between immunoreactivity of GPR30 and that of ER in cervical AIS and adenocarcinoma lesions (Spearman's correlation, r=-0.324, p=0.017). ROC curve analysis revealed that immunoreactivity of GPR30 successfully distinguished neoplasms from non-neoplastic glands with high specificity (100%) and sensitivity (75.5%). GPR30 positivity was significantly correlated with histological type (p=0.009), tumor diameter (p=0.003), tumor size (p<0.001), lymphovascular infiltration (p=0.005) and UICC stage (p<0.001). ER expression was correlated only with tumor factor (p=0.047). GPR30-high patients had poor prognosis with a significantly shorter overall survival (OS) period (p=0.0309). GPR30 expression is a potential diagnostic and prognostic marker.

Key words: Cervical adenocarcinoma, Estrogen receptor, G protein-coupled receptor 30 (GPR30), G protein-coupled estrogen receptor 1 (GPER1), Invasion front, prognosis

Introduction

The incidence of uterine cervical adenocarcinoma has been increasing worldwide, predominantly in young women, despite the fact that the incidence of squamous cell carcinoma (SCC) has been decreasing (Gien et al., 2010; Fujiwara et al., 2014; Ferlay et al., 2015; van der Horst et al., 2017; Akimoto et al., 2018). It has been shown that adenocarcinoma has a worse prognosis than that of SCC at the same stage and with the same tumor size. The main reasons for the worse prognosis are a higher rate of metastases (Nakanishi et al., 2000) and resistance to radiotherapy and chemotherapy (Lee et al., 2015; Takeuchi, 2016; Yokoi et al., 2017). Accurate initial diagnosis has important implications related to appropriate triage of high-risk patients, but many studies have shown that diagnosis of a cervical gland lesion is more difficult than that of a squamous cell lesion

(Soonthornthum et al., 2011; McCluggage, 2013; Akimoto et al., 2016; Ueda et al., 2017). Therefore, a new treatment strategy is needed to improve the outcome of cervical adenocarcinoma.

Estrogen is an important steroid hormone that plays a key role in development and function of the female reproductive tract. Estrogen binds to its receptor to activate downstream signaling pathways (Chuffa et al., 2017). Estrogen receptors (ERs) include ERα, ERβ, and G protein-coupled receptor 30 (GPR30). Classical types ERα and ERβ act as nuclear receptors for estrogen, whereas GPR30 acts as a cell surface receptor to mediate rapid non-genomic signaling of estrogen (Prossnitz and Barton, 2014).

GPR30, formerly known as an orphan member of the G protein-coupled receptor superfamily, is expressed on the cell membrane and in the cytoplasm, and has been linked to specific estradiol binding. (Prossnitz and Maggiolini, 2009). In human tissues, GPR30 is expressed in reproductive, nervous, immune, cardiovascular and skeletal systems, and maintains the functions of those systems (Prossnitz and Barton, 2011). Recently, it has been reported that GPR30 mediates the rapid non-genomic signaling of estradiol in a variety of estrogen-responsive cancer cells. GPR30 is expressed in malignant tumors such as breast cancer and endometrial cancer and that GPR30 is associated with tumor cell proliferation, migration and invasion (Wang et al., 2010). On the other hand, there is only one report about the relationship between cervical cancer and GPR30 in surgical specimens of patients (Friese et al., 2018).

In this study, we examined the immunohisto-chemical expression of GPR30 and classical ER in adenocarcinoma of the uterine cervix and then analyzed relationships between expression of the receptors and clinicopathological parameters.

Materials and methods

Patients and specimens

Surgical specimens obtained from 53 cases of cervical adenocarcinoma (33 endocervical type endocervical adenocarcinoma, 4 intestinal type mucinous carcinoma, 3 gastric type mucinous carcinoma (or minimal deviation type), 3 villoglandular type and 10 adenocarcinoma *in situ* "AIS") during the period from 2004 to 2012 were retrieved from the pathology file of Sapporo Medical University Hospital, Sapporo, Japan. As controls, adjacent non-neoplastic regions were examined as normal tissues (n=44). The histological type was based on the WHO classification of tumors of the uterine cervix, 4th edition. Fifty-three cases were classified by tumor staging on the basis of the Union for International Cancer Control (UICC) stage classification (7th edition). This study was approved by the Institutional Review Board of Sapporo Medical University (IRB study number: 302-197). Written informed consent was obtained from each patient who participated in the investigation.

Clinicopathological data

We retrospectively collected clinicopathological data for age, histological type, tumor size, lymph node metastasis, lymphovascular infiltration, UICC stage, relapse-free survival (RFS) and overall survival (OS). Clinicopathological features are summarized in Table 1.

Immunohistochemical staining of surgical specimens

Hematoxylin and eosin (H&E)-stained slides from all cases were reviewed to select representative sections. New sections were prepared from paraffin blocks of formalin-fixed surgical specimens and were immunohistochemically stained. Sections were deparaffinized, rehydrated, and moistened with phosphate-buttered saline (PBS) (pH 7.4). Antigen retrieval was performed by using a microwave in Tris-EDTA (pH 9.0) for 30 min. Primary antibodies were anti-GPR30 (PA5-28647, x100, Thermo Fisher Scientific, Rockford, IL) and anti-ER (SP1, x200, Abcam, Cambridge, UK). Secondary antibody reaction and detection of immunoreactivity were performed by using the Dako Real™ EnVision™ detection system (DAKO, Carpinteria, CA).

Immunohistochemical analysis

Immunoreactivity was manually scored as the percentage of positive cells for semiquantitative analysis. Epithelial cells with membrane and cytosolic staining were judged as positive cells. The intensity of staining was assessed as negative (0), weak (1), moderate (2) or strong (3). The proportions of staining of positively stained tumor cells were recorded as 0 (no staining), 1 (1-10%), 2 (11-20%), 3 (21-30%), 4 (31-40%), 5 (41-50%), 6 (51-60%), 7 (61-70%), 8 (71-80%), 9 (81-90%) and 10 (91-100%). Because neoplasm heterogeneity caused varying degrees of immuno-reactivity in the slides, we used an immunoreactive score (IRS) (i.e., intensity 3 × proportion 10=immunoreactive score 30, scale of 0 to 30) for improvement in accuracy (Keira et al., 2015). When evaluating the slides, the pathologists (A.T, M.M and M.O) were blinded to the clinical data. Discordant cases were discussed, and a consensus was reached.

Statistical analysis

Statistical analysis was performed using Pearson's chi-squared test, Fisher's exact test, the Kruskal-Wallis test, Spearman's rank correlation coefficient and the logrank test. Kaplan-Meier curves were generated for high expression group (IRS>10) and low expression group (IRS≤10). All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan) (Kanda, 2013), which is a

graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics.

Results

Patient characteristics and clinicopathological characteristics

Patient characteristics and clinicopathological characteristics are summarized in Table 1. The study population consisted of 53 patients with an age range of 25 to 79 years. The median age of the patients was 43 years. All cases were classified by tumor staging on the basis of UICC stage classification (7th edition): stage 0 (n=10), stage IA (n=6), stage IB (n=26), stage IIA (n=4), stage IIB (n=1), and stage IIIB (n=6). The frequencies of lymph node metastasis and lymphovascular infiltration were 11.3% and 28.3%, respectively.

Immunohistochemistry of GPR30 and ER in cervical adenocarcinoma

Immunohistochemistry was performed on surgical specimens using anti-GPR30 and anti-estrogen receptor (ER) antibodies. First, we examined the expression of GPR30. In normal cervical glands (CGs), almost no expression of GPR30 was observed (Fig. 1D). In contrast, strong expression of GPR30 was observed on the whole membrane and in the cytoplasm in adenocarcinoma (ADC), and the expression was especially strong at the invasion front in more than half of the GPR30-immunoreactive ADC cases (Fig. 1F,J,K). There was no difference in the expression of GPR30 among histological subtypes of ADC (data not shown). Adenocarcinoma *in situ* (AIS) showed weak to moderate expression on the whole membrane and cytoplasm (Fig. 1E). In contrast to the expression pattern of GPR30, nuclear staining of ER was strong in CGs, whereas it was almost absent in most of the AIS and ADC cases (Fig. 1G-I).

To maintain high levels of reproducibility and accuracy of immunohistochemical evaluation, we defined a parameter, designated as immunoreactive score (IRS), which was calculated by multiplying intensity (4 grades) and proportion (11 grades) of immunoreactivity (Keira et al., 2015). Mean±SD values of IRS of GPR30 were 0.0±0.2 in CGs, 2.8±3.7 in AIS and 11.2±10.0 in ADC (Fig. 2A). GPR30 was expressed at significantly higher levels in AIS (p<0.001) and ADC (p<0.001) than in CG. In addition, the IRS of GPR30 in ADC was significantly higher than that of GPR30 in AIS (p<0.001). The invasion front was clearly GPR30-positive in 12 (63.2%) of 19 cases with high IRS of GPR30 (11 or more). Mean±SD values of IRS of ER were 26.0±6.8 in CGs, 3.5±2.3 in AIS and 4.1±7.0 in ADC (Fig. 2B). IRSs of ER were significantly lower in AIS (p<0.001) and ADC (p<0.001) than that in CGs.

However, no significant difference was found between the IRS of ER in AIS and that of ER in ADC (p=0.700) (Fig. 2B). Fig. 2C shows scatter plots of IRSs of GPR30 and ER. There was a weak but statistically significant negative correlation between IRS of GPR30 and that of ER in cervical AIS and adenocarcinoma lesions (Spearman's correlation, r=-0.324, p=0.017).

Diagnostic value of GPR30

To assess the utility of immunohistochemistry of GPR30 as a diagnostic marker, we performed receiver operator characteristic curve (ROC) analysis. The area under the receiver operator characteristic curve (AUC) was 0.884 [95% CI, 82.6 to 94.2]. A cut-off value of two produced the highest accuracy; specificity and sensitivity were 100% and 75.5%, respectively (Fig. 2D). These results indicated that immunohistochemistry of GPR30 successfully distinguished neoplasms from non-neoplastic cervical glands.

Correlations between expression of GPR30 and clinicopathological features of cervical adenocarcinoma

We next examined possible relationships between the expression status of GPR30 and clinicopathological parameters. At a cutoff value of 10, as shown in Table 2, high GPR30 expression was significantly correlated with

Table 1. Clinicopathological features of cervical adenocarcinomas.

| Patients (n=53)	
Age (range, median)	25-79, 43
Histological type	
AIS (Adenocarcinoma *in situ*)	10
Adenocarcinoma	43
Endocervical type (MuE)	33
Intestinal type (MuI)	4
Minimul deviation type (MuM)	3
Villoglandular type (MuV)	3
Tumor stage (UICC)	
0	10
IA	6
IB	26
IIA	4
IIB	1
IIIA	0
IIIB	6
Diameter	
AIS only	10
≤40mm	31
>40mm	12
Lymph node metastasis	
Negative	47
Positive	6
Lymphovascular infiltration	
Negative	38
Positive	15
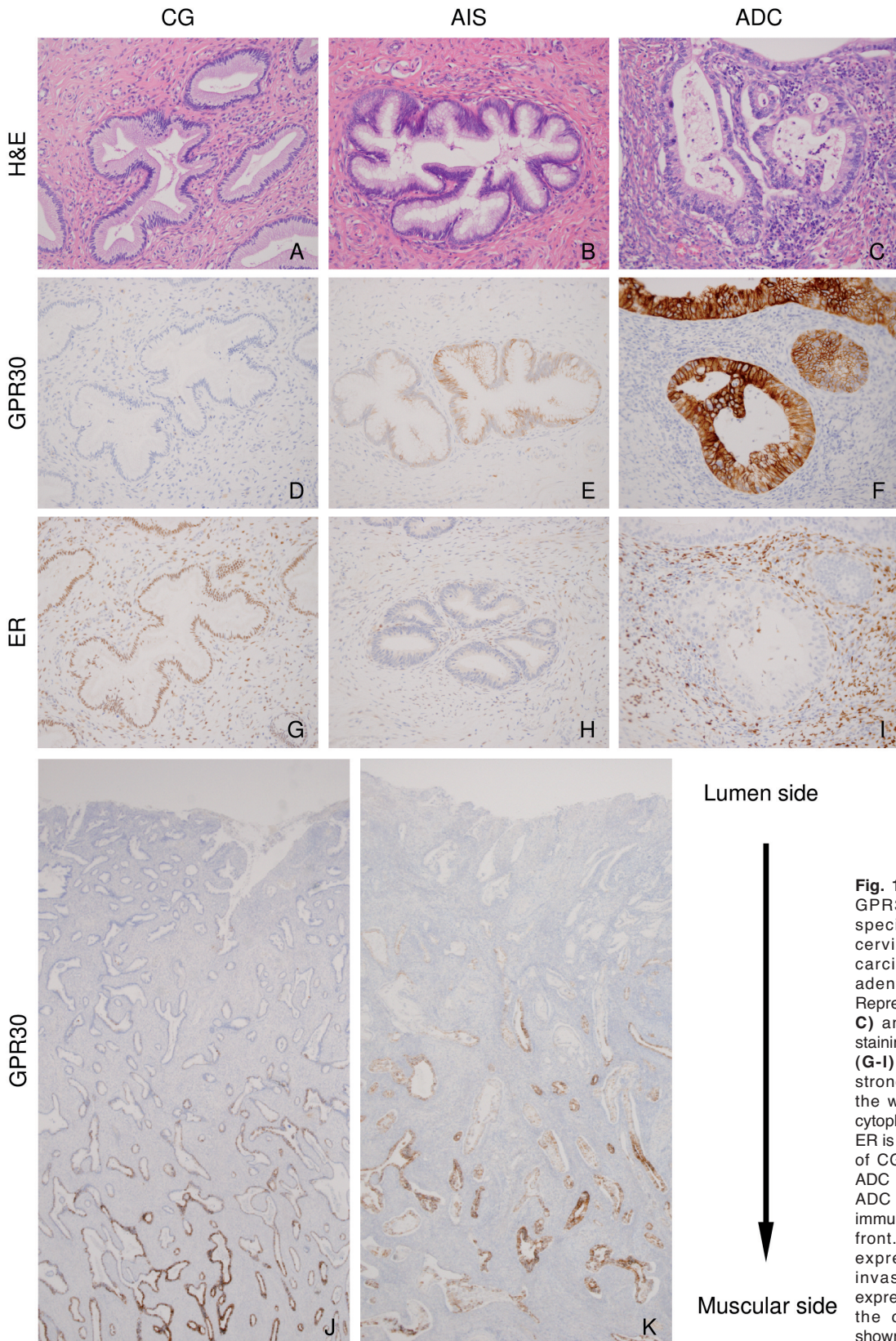
Fig. 1. Immunohistochemistry of GPR30 and ER in surgical specimens of non-neoplastic cervical glands (CG), adeno-carcinoma *in situ* (AIS), and adenocarcinoma (ADC). **(A-I)** Representative H&E staining **(A-C)** and immunohistochemical staining of GPR30 **(D-F)** and ER **(G-I)** are shown. GPR30 is strongly expressed throughout the whole cell membrane and cytoplasm of AIS and ADC cells. ER is strongly expressed in nuclei of CG cells but not in AIS and ADC cells. **(J, K)** Representative ADC cases with strong GPR30 immunoreactivity at the invasion front. Some cases with strong expression of GPR30 at the invasion front despite weak expression or no expression in the cervical lumen side are shown. x 20.

histological type (p=0.009), tumor diameter (p=0.003), tumor size (p<0.001), lymphovascular infiltration (p=0.005) and UICC stage (p<0.001); however, there was no significant correlation between GPR30 expression and patient's age (p=0.398) or lymph node metastasis (p=0.170). ER expression was correlated only with tumor size (p=0.047).

Correlation between expression of GPR30 and survival of patients with cervical adenocarcinoma

The relationships of the expression of GPR30 and ER in cervical adenocarcinoma with relapse-free survival (RFS) and overall survival (OS) were assessed by using the Kaplan-Meier method. Patients with a higher GPR30 immunoreactive score (11 or more) had a worse prognosis, and the OS period of patients with a higher GPR30 score was significantly shorter than that of patients with an IRS smaller than 11 (Fig, 3A,B). In contrast to GPR30, patients with a lower IRS of ER (smaller than 10) had a weak tendency for a worse prognosis than that of patients with a high score (Fig. 3C,D).

Discussion

This is the first study to evaluate the importance of membrane-type estrogen receptor GPR30 expression in cervical adenocarcinoma, referring classical nuclear ER expression.

We found that the expression level of GPR30 was significantly increased in AIS and cervical adenocarcinoma compared to its level in non-neoplastic cervical glands. ROC curve analysis revealed that immunohistochemistry of GPR30 can differentiate non-neoplastic glands from AIS/adenocarcinoma with high specificity and sensitivity, suggesting that it is a useful diagnostic marker. For clinical application, further prospective and retrospective multicenter studies and studies using biopsy specimens are needed.

Generally, cervical adenocarcinoma has been thought to be an estrogen-insensitive disease because classical nuclear ER expression was negative or weak in the malignancy and was not associated with the prognosis or clinicopathological parameters of uterine cervical cancers (Fujiwara et al., 1997; Bodner et al., 2010). Actually, we also found that the immunoreactive
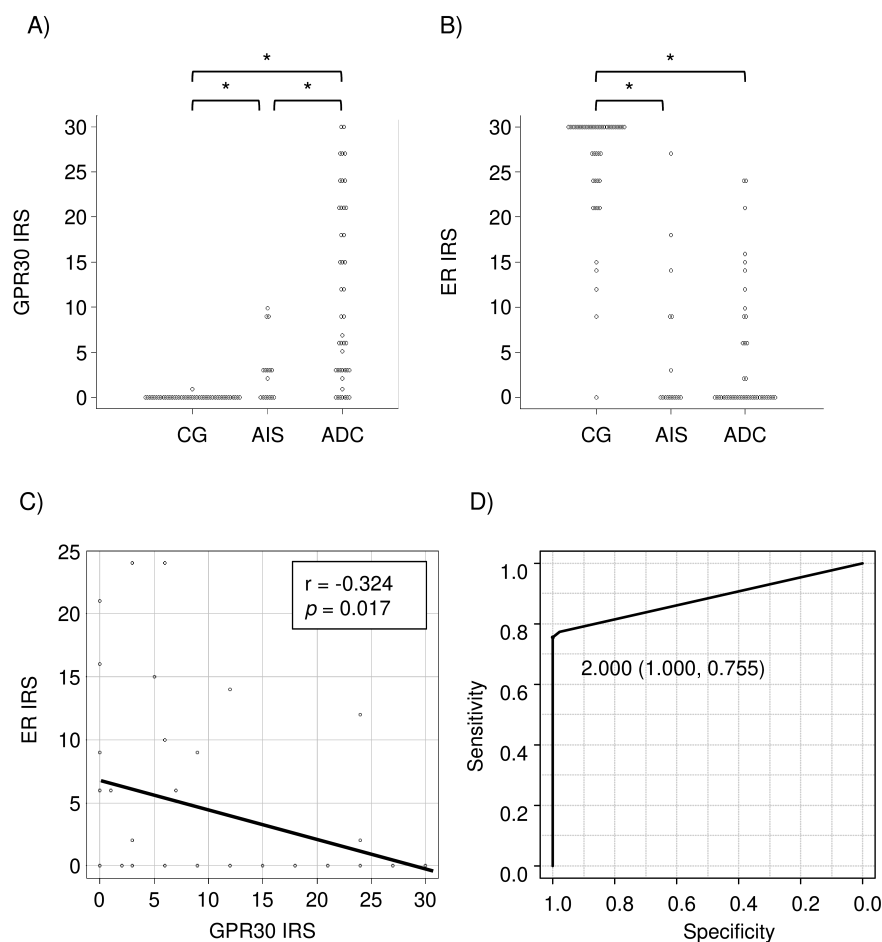

Fig. 2. Analysis of immunoreactive scores of GPR30 and ER in surgical specimens. **A, B.** Scatter plots of the immunoreactive scores (IRSs) of GPR30 and ER in each histological type. IRSs of GPR30 in AIS and ADC were significantly higher than that in CG, whereas IRSs of ER in AIS and ADC were significantly lower than that in CG, *p<0.001, Student's t-test. **C.** Spearman's correlation between IRS of GPR30 and IRS of ER. Higher GPR30 expression level indicates lower ER expression level (n=53). The black line shows the regression line (r=-0.324, p=0.017). **D.** ROC analysis showed that the highest accuracy (specificity, 100%; sensitivity, 75.5%) was obtained at a cut-off value of two to distinguish neoplasms (AIS/ADC) from non-neoplastic cervical glands. CG: non-neoplastic cervical gland, AIS: adenocarcinoma *in situ*, ADC: adenocarcinoma.

Table 2. The correlation analyses between the expression of estrogen receptors and clinicopathological features.

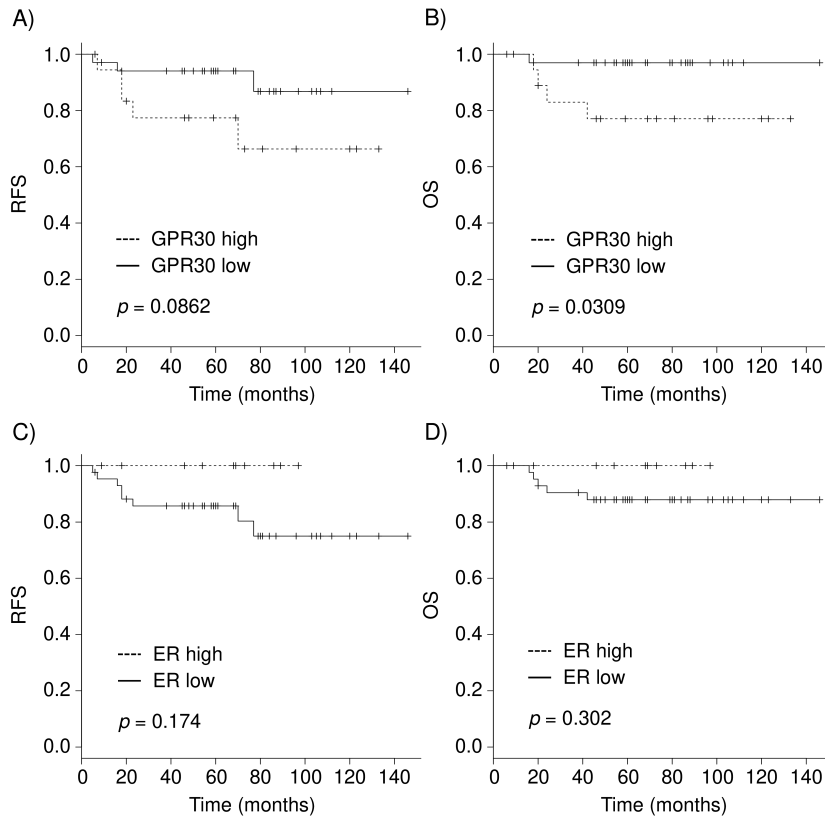|		GPR30			ER		
	N	Low	High	P value	Low	High	P value
Age							
under median (<=43)	27	19	8	0.398	20	7	0.293
over median (>43)	26	15	11		23	3	
Histology							
AIS	10	10	0	0.009	10	0	0.178
Adenocarcinoma	43	24	19		33	10	
Diameter							
AIS	10	10	0	0.003	10	0	0.263
<=40 mm	31	20	11		24	7	
>40 mm	12	4	8		9	3	
Tumor size							
pT0	10	10	0	<0.001	10	0	0.047
pT1	33	23	10		23	10	
pT2	9	1	8		9	0	
pT3	1	0	1		1	0	
Lymph node metastasis							
Negative	47	32	15	0.170	38	9	1.000
Positive	6	2	4		5	1	
Lymphovascular infiltration							
Negative	38	29	9	0.005	31	7	1.000
Positive	15	5	10		12	3	
Tumor stage (UICC)							
0	10	10	0	<0.001	10	0	0.159
I	32	22	10		23	9	
II	5	0	5		5	0	
III	6	2	4		5	1	

Fig. 3. Kaplan-Meier curves of relapse-free survival (RFS) and overall survival (OS) of patients with cervical adenocarcinoma according to the expression of GPR30 or ER. Patients with high GPR30 expression had worse OS than did patients with low GPR30 expression, as shown by the log-rank test (p=0.0309).

score of ER was significantly smaller in cervical adenocarcinoma than in non-neoplastic glands (Fig. 2B), and the rate of ER expression in cervical adenocarcinoma cases was only 18.8% (10/53), being similar to previously reported rates of 20-39% (Masood et al., 1993; Ghandour et al., 1994; Fujiwara et al., 1997; Bodner et al., 2010). On the other hand, we found that the expression level of membrane-type estrogen receptor GPR30 was significantly increased in AIS and cervical adenocarcinoma compared to its level in non-neoplastic cervical glands (Fig. 2A), suggesting that signaling can occur in response to estrogen binding to GPR30 in cervical adenocarcinoma. The idea of possible estrogen/GPR30 signaling is supported by the results of our recent study showing that the expression of tumor-promoting cell surface protein claudin-1 is upregulated by estrogen/GPR30 signaling in cervical adenocarcinoma cell lines (Akimoto et al., 2018). The possibility for estrogenic disease progression provides a new insight into the etiology of cervical adenocarcinoma. A high level of serum estrogen or a side effect of hormonal therapy should be reexamined from the viewpoint of cervical adenocarcinoma development as reported for the development of several cancers (Bissett et al., 1994; Sismondi et al., 1994; Vivacqua et al., 2006, 2012; Mountzios et al, 2011; Ryu et al., 2012; Yi et al., 2014; Brown and Hankinson, 2015).

Interestingly, the expression of ER and that of GPR30 showed a weak but statistically significant negative correlation in cervical adenocarcinoma (Fig. 2C). The correlation might reflect a sort of 'estrogen receptor switching', enabling cells to promote rapid estrogen signaling via the cell surface receptor GPR30 toward tumor progression.

In the present study, GPR30 expression was correlated with some clinicopathological features and with poor prognosis of cervical adenocarcinoma. Recently, GPR30 has been reported to be involved in tumor progression of several gynecological cancers including breast cancer, endometrial cancer and ovarian cancer. GPR30 expression promotes proliferation, migration and invasion of tumor cells (Filardo et al., 2000; Qian et al., 2016) and is associated with a poor prognosis (Filardo et al., 2006; Smith et al., 2007, 2009). Mechanistically, those tumor-promoting roles were attributed to cell surface receptor function of GPR30 in rapid response to estrogen signaling (Prossnitz and Maggiolini, 2009). Interestingly, we found that 63.2% (12/19) of high GPR30 ADC cases showed strong GPR30 positivity at the invasive front, where the most aggressive tumor cells presumably exist (Fig. 1J). Overexpression of GPR30 at the invasion front suggests that GPR30 has some roles in proliferation, invasion and migration ability of cervical adenocarcinoma cells like the roles of ZEB2, GLUT-1 and TFF1 in several cancers (Ohba et al., 2010; Kahlert et al., 2011; Sunagawa et al., 2017). As in other cancers, a high level of GPR30 may also serve as a cell surface estrogen receptor to promote

cervical adenocarcinoma progression. Recently, we reported that a tumor-promoting cell surface protein claudin-1 is one of the downstream molecules regulated by estrogen/GPR30 signaling and contributes to malignant potentials of cervical adenocarcinoma cells (Akimoto et al., 2018).

There has been only one study on GPR30 expression in cervical cancer, and squamous cell carcinoma (SCC) and adenocarcinoma (ADC) were not separated but were collectively analyzed in that study (Friese et al., 2018). Friese et al. reported that cytoplasmic GPR30 staining showed a negative correlation with tumor grade and that GPR30 staining was associated with improved overall and recurrence-free survival in patients with early-stage cervical cancer (FIGO I). Their conclusion regarding GPR30 is almost opposite to the conclusion drawn from the results of our study, possibly due to differences in sample composition (SCC and ADC vs ADC only) and antibodies.

In conclusion, a high expression level of GPR30 is a potential biomarker and predicts poor prognosis in patients with cervical adenocarcinomas. Our results also suggest that cervical adenocarcinoma is an estrogen-sensitive tumor, and estrogen/GPR30 signaling might become a therapeutic target in cervical adenocarcinoma.

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