



















Original Article



# High expression of folate receptor alpha is associated with poor prognosis in patients with cervical cancer

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## ABSTRACT

**Objective:** Folate receptor  $\alpha$  (FR $\alpha$ ) is a membrane protein expressed in various solid tumors but has limited expression in normal cells. Therefore, FR $\alpha$  is an attractive target for cancer treatment. This study aimed to investigate the relationship between FR $\alpha$  expression and the clinicopathological characteristics and survivals of cervical cancer.

**Methods:** This retrospective study included patients with cervical cancer who underwent primary surgery between 2000 and 2020 at our institution. Immunohistochemical staining of FR $\alpha$  was performed using an anti-folate-binding protein/FBP antibody. FR $\alpha$ -positive staining was defined as  $\geq 5\%$  of tumor staining and FR $\alpha$ -high as  $\geq 50\%$  tumor staining with  $\geq 2+$  intensity. The association between FR $\alpha$  expression and survival was assessed using multivariate Cox regression analysis, adjusting for established prognostic factors.

**Results:** Overall, 123 patients were identified, and 140 tumor samples, including 17 paired primary and metastatic samples, were evaluated. As histological types, 67 patients had squamous cell carcinoma (SCC), and 56 patients had non-SCC. All primary tumors were FR $\alpha$ -positive. High FR $\alpha$  expression was observed in 25% of the cases and differed according to histology (SCC vs. non-SCC, 14.9% vs. 37.5%,  $p=0.004$ ). FR $\alpha$  expression was significantly higher in metastatic tumors than in primary (170 [IQR, 140–205] vs. 125 [IQR, 110–150],  $p=0.0006$ ). High FR $\alpha$  expression was significantly associated with worse overall survival (hazard ratio, 6.73; 95% confidence interval, 2.21–20.53;  $p=0.001$ ).

**Conclusion:** In cervical cancer, FR $\alpha$  expression was elevated in metastatic tumors and high expression was associated with a worse prognosis. Our study supports the development of FR $\alpha$ -targeted therapy for advanced cervical cancer.

**Keywords:** Folate Receptor Alpha; Cervical Cancer; Biomarkers

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#### Conflict of Interest

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#### Synopsis

The association between folate receptor  $\alpha$  (FR $\alpha$ ) and cervical cancer remains unclear. High FR $\alpha$  expression was observed in 25% of the patients, which was more frequent in non-squamous histology. The FR $\alpha$  expression was significantly higher in the metastatic than in the primary tumors. FR $\alpha$  was an independent prognostic factor for poor overall survival.

## INTRODUCTION

Cervical cancer is the fourth most common cancer among women worldwide [1]. Although the incidence of cervical cancer has been decreasing in Western countries due to the widespread use of vaccines against high-risk human papillomavirus (HPV), the incidence is still high in Asia, including Japan [2,3]. Squamous cell carcinoma (SCC) is the predominant histological type of cervical cancer, accounting for 80%–90% of cervical cancers. Other common types are adenocarcinoma (AC) and adenosquamous carcinoma (ASC), found in approximately 10%–25% and 5%–6% of the cases, respectively. No specific treatment has been established for each histological type. The standard treatment for metastatic or recurrent cervical cancer is systemic chemotherapy, but the prognosis is poor, with a median survival of 17 months [4,5]. When patients become refractory to first-line platinum-based chemotherapy, second-line and subsequent treatments have limited efficacy [6]. Therefore, novel treatment strategies need to be developed. Recent advances in molecular targeted therapy have improved the prognosis of other cancer types [7], and it is crucial to investigate new therapeutic targets to improve the prognosis of cervical cancer.

Folate receptor  $\alpha$  (FR $\alpha$ ) is a 38–40 kDa glycosyl-phosphatidylinositol-anchored membrane protein [8] encoded by *FOLR1*. FR $\alpha$  is considered to play a role in cell migration and invasion, and overexpression of this receptor is associated with tumor progression in preclinical models [9]. FR $\alpha$  is expressed over the entire surface of tumor cells in various cancer types, including ovarian cancer, triple-negative breast cancer (TNBC), endometrial cancer, and non-small cell lung cancer (NSCLC) [10-12]. In contrast, non-malignant tissues exhibit limited FR $\alpha$  expression. Therefore, FR $\alpha$  has been investigated as a potential target for cancer treatment, and various therapeutic developments are underway, including antibody-drug conjugates (ADCs), small molecules, and chimeric antigen receptor (CAR)-T therapy [13-15]. Mirvetuximab soravtansine, an ADC comprising an FR $\alpha$ -binding antibody, a cleavable linker, and the potent tubulin-targeting agent maytansinoid DM4, has demonstrated a tumor response in relapsed ovarian cancer with high FR $\alpha$  expression [16]. An FR $\alpha$ -targeted thymidylate synthase inhibitor has also shown an objective response in FR $\alpha$ -overexpressing ovarian cancer [17]. These FR $\alpha$ -targeted treatments may improve the prognosis of FR $\alpha$ -overexpressing tumors.

To date, no studies have evaluated the association between FR $\alpha$  expression and the clinicopathological features and prognosis of cervical cancer. A previous study by Liu et al. [18] has shown that 40% of SCCs exhibit moderate or high FR $\alpha$  expression. However, FR $\alpha$  expression in non-SCC patients and its prognostic role remain unclear. In this study, we aimed to evaluate FR $\alpha$  expression in various histological types of cervical cancer and its association with prognosis. We also compared FR $\alpha$  expression between primary and paired metastatic tumors to examine the stability of FR $\alpha$  expression status during disease progression.

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#### Author Contributions

Conceptualization: Y.S., K.Y., Y.H.; Data curation: Y.S.; Formal analysis: Y.S., K.Y.; Methodology: Y.H.; Project administration: K.Y.; Supervision: Y.K.; Writing - original draft: Y.S., K.Y., Y.H.; Writing - review & editing: T.S., K.R., N.T., S.T., S.K., S.A., O.H.S., T.M., N.E., U.M., I.M., K.T., F.Y., O.Y., Y.K.

## MATERIALS AND METHODS

### 1. Study population

We identified patients who underwent surgery as a primary treatment at the National Cancer Center Hospital (Tokyo, Japan) between January 2000 and December 2020. Among these patients, we further identified those who subsequently developed metastatic disease and underwent tumor biopsy for paired metastatic tumors. Patients with unavailable or insufficient primary tumor tissue were excluded from the study. We retrospectively collected clinical and pathological data, such as age, histology, clinical stage according to the International Federation of Gynecology and Obstetrics (FIGO) 2008 system [19], lymph node metastasis, adjuvant treatment, and survival time after surgery. All cases were reviewed, and pathological diagnoses were confirmed by at least two gynecological pathologists. Furthermore, given that the study period was approximately 20 years, the permanent slides of all cases were microscopically reviewed, and the final diagnoses were confirmed for the present study according to the 2014 World Health Organization classification of cervical cancer [20]. In this study, we separated usual-type endocervical adenocarcinoma and gastric-type adenocarcinoma (GAS) for subsequent analysis because these two types of adenocarcinoma reportedly have quite different etiologies and clinicopathological features [21,22]. This study was approved by the Institutional Review Board of the National Cancer Center (Tokyo, Japan) (No. 2014-393). Written informed consent was waived because of the retrospective design.

### 2. Immunohistochemical staining and evaluation

Hematoxylin and eosin-stained slides for each case were reviewed to obtain representative sections. New 4- $\mu$ m-thick sections were prepared from formalin-fixed paraffin-embedded surgical specimens. FR $\alpha$  expression was evaluated by immunohistochemical staining using rabbit anti-folate binding protein/FBP antibody (ab67422; Abcam, Cambridge, UK) at a dilution of 1:200. Antigen retrieval was achieved in Target Retrieval Solution (pH 9) using a PT Link machine (Dako, Glostrup, Denmark). Immunohistochemical staining was performed using the Dako Autostainer Link 48 and EnVision Flex Mini kit (Dako), according to the manufacturer's protocols. Briefly, deparaffinization, rehydration, and antigen retrieval (97°C, 20 minutes) were performed using PT-Link (Dako) in EnVision™ FLEX Target Retrieval Solution High pH 9.0 (Dako) for the antibody to FBP. Endogenous peroxidase was blocked using EnVision™ FLEX Peroxidase-Blocking Reagent (Dako) (5 minutes). Slides were then stained with Dako Link 48 Autostainer Immunostaining System (Agilent Technologies, Santa Clara, CA, USA) at room temperature for 60 min. EnVision Flex Mini-Kit HRP (K8023; Dako) was applied for 30 min; then, staining was visualized with EnVision Flex DAB-substrate chromagen (EnVision Flex Mini-Kit; Dako), which had been applied for 10 minutes. Slides were counterstained with hematoxylin (#SK203; Dako), dehydrated through graded ethanol solutions, cleared with xylene, and coverslipped. The H-score of FR $\alpha$  expression was calculated using the following formula:  $3X+2Y+Z$ , where X, Y, and Z are the proportions of tumor cells showing strong (3+), moderate (2+), and weak (1+) staining intensities, respectively. The maximum H-score was 300 (strong staining of all tumor cells), and the minimum was 0 (no staining of any tumor cells). The immunohistochemistry results were defined as FR $\alpha$ -positive if  $\geq 5\%$  of tumor cells had FR $\alpha$  expression at  $\geq 1+$  intensity. The tumor tissues were scored for FR $\alpha$  expression as follows: high  $\geq 50\%$  of tumor cells  $\geq 2+$  staining intensity and low  $< 50\%$  of tumor cells  $\geq 2+$  staining intensity.

### 3. Association between *FOLR1* mRNA expression and histological types of cervical cancer using The Cancer Genome Atlas (TCGA) dataset

*FOLR1* mRNA expression data were obtained from the cBioPortal [23,24]. Of mRNA expression data available from 306 patients with cervical cancer, data for a total of 243 patients with either SCC (n=221) or AC (n=22) were analyzed. An analysis was performed on the log-transformed RNA-Seq by Expectation-Maximization V2-normalized data.

### 4. Statistical analysis

Continuous variables were reported as median, range, and interquartile range (IQR) and were compared using the Mann-Whitney U test. Categorical variables were reported as numbers and percentages and compared using the chi-square test. Disease-free survival (DFS) and overall survival (OS) were estimated using the Kaplan-Meier method. The log-rank test was used to compare the survival between the groups. DFS was defined as the time from surgery to the first relapse or death from any cause. OS was defined as the time from surgery to the death from any cause. We used a Cox proportional hazards model for univariate and multivariate analyses to identify the prognostic value of high FR $\alpha$  expression. All tests were two-tailed, and the significance level was set at  $\alpha=0.05$ . Statistical analyses were performed using STATA (version 15.1; StataCorp, College Station, TX, USA) and GraphPad Prism version 8.0 (GraphPad Software, San Diego, California, USA).

## RESULTS

### 1. Patient characteristics

A total of 123 patients were included in the analysis. Among them, 17 patients had paired metachronous metastatic tumor samples for which FR $\alpha$  expression could be assessed. Patient characteristics are shown in **Table 1**. The median age was 45 years (range, 26–72 years). There were 67 patients (54.5%) with SCC, 27 patients (22.0%) with AC, all of which had usual-type endocervical adenocarcinoma, 13 patients (10.6%) with ASC, 12 patients (9.8%) with GAS, and 4 patients (3.3%) with neuroendocrine carcinoma. Ninety-six patients (78%) had FIGO stage I disease while 27 (22%) had FIGO stage II disease. Lymph node metastasis was present in 45 patients (36.6%). For treatment, 44 patients (35.8%) received postoperative radiotherapy (RT), and 13 patients (10.6%) received postoperative concurrent chemoradiotherapy (CCRT).

### 2. Association between FR $\alpha$ expression and clinicopathological features

FR $\alpha$  expression was detected in all primary tumor samples. The median H-score of the primary tumor samples was 125 (IQR, 110–150). High FR $\alpha$  expression was found in 25% (31/123) of the primary tumor samples. **Fig. 1A and B** show representative images of FR $\alpha$  expression in SCC and AC, respectively. High FR $\alpha$  expression was associated with non-SCC (21 [67.7%] vs. 35 [38.0%]) and lymphovascular space involvement (LVSI) (25 [80.7%] vs. 52 [56.5%]) compared with low FR $\alpha$  expression. Other clinicopathological factors, including age (<45 vs.  $\geq$ 45 years), tumor size ( $\leq$ 4 vs. >4 cm), stage (FIGO stage I vs. II), lymph node metastasis (positive vs negative), and adjuvant RT/CCRT (yes vs. no), were not significantly associated with high FR $\alpha$  expression.

High FR $\alpha$  expression was more frequent in non-SCC than in SCC histology (non-SCC vs. SCC, 37.5% [21/56] vs. 14.9% [10/67];  $p=0.004$ ). Of note, 54% (7/13) of the patients with ASC and 42% (5/12) of those with GAS had high FR $\alpha$  expression (**Table 2**). According to TCGA

**Table 1.** Patient characteristics

Characteristics	Total (n=123)	FR $\alpha$ -high (n=31)	FR $\alpha$ -low (n=92)	p-value
Age (yr)	45 (26–72)	44 (30–67)	46 (26–72)	0.500
Histological types				0.009
SCC	67 (54.5)	10 (32.3)	57 (62.0)	
AC	27 (22.0)	9 (29.0)	18 (19.6)	
GAS	12 (9.8)	5 (16.1)	7 (7.6)	
ASC	13 (10.6)	7 (22.6)	6 (6.5)	
NEC	4 (3.3)	0 (0)	4 (4.4)	
FIGO stage (2008)				0.040
IA1	2 (1.6)	2 (6.5)	0 (0)	
IB1	62 (50.4)	13 (41.9)	49 (53.3)	
IB2	32 (26.0)	7 (22.6)	25 (27.2)	
IIA1	5 (4.1)	0 (0)	5 (5.4)	
IIA2	6 (4.9)	2 (6.5)	4 (4.3)	
IIB	16 (13.0)	7 (22.6)	9 (9.8)	
Tumor size				0.110
$\leq$ 4 cm	67 (54.5)	13 (41.9)	54 (58.7)	
$>$ 4 cm	56 (45.5)	18 (58.1)	38 (41.3)	
Lymph nodes metastasis				0.780
Positive	45 (36.6)	12 (38.7)	33 (35.9)	
Negative	78 (63.4)	19 (61.3)	59 (64.1)	
LVSI				0.016
Positive	77 (62.6)	25 (80.7)	52 (56.5)	
Negative	46 (37.4)	6 (19.4)	40 (43.5)	
Adjuvant RT/CCRT				0.270
Yes	57 (46.3)	17 (54.8)	40 (43.5)	
No	66 (53.7)	14 (45.2)	52 (56.5)	

Values are presented as median (range) or number (%).

AC, adenocarcinoma; ASC, adenosquamous carcinoma; CCRT, concurrent chemoradiotherapy; FIGO, International Federation of Gynecology and Obstetrics; FR $\alpha$ , folate receptor  $\alpha$ ; GAS, gastric-type adenocarcinoma; LVSI, lymphovascular space involvement; NEC, neuroendocrine carcinoma; RT, radiotherapy; SCC, squamous cell carcinoma.

dataset, *FOLR1* mRNA expression was higher in AC than in SCC (log fold change  $-4.76$ ,  $p < 0.001$ ; **Fig. 1C**).

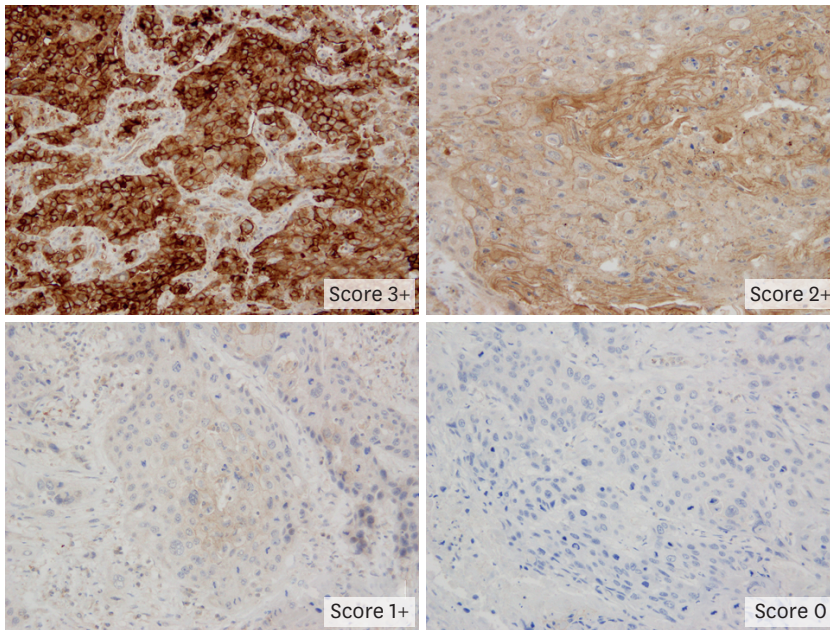
### 3. FR $\alpha$ expression in primary and metastatic tumors

First, we compared FR $\alpha$  expression between the primary and metastatic tumor samples in the total population. The median H-score of FR $\alpha$  expression in metastatic tumors was significantly higher than that in primary tumors (primary vs. metastatic, 125 [IQR, 110–150] vs. 170 [IQR, 140–205];  $p = 0.0006$ ) (**Fig. 2A**). Twelve of the 17 (70%) metastatic tumors showed high FR $\alpha$  expression. Next, we analyzed FR $\alpha$  expression in patients with primary and paired metastatic tumor samples ( $n = 17$ ). The H-score of FR $\alpha$  expression in the paired metastatic tumors was higher than that in the primary tumors in 13/17 (76.5%) of the patients. Among the 13 patients with low FR $\alpha$  in the primary tumor, 8 patients (61.5%) had high FR $\alpha$  in the paired metastatic tumor (**Fig. 2B**). None of the patients were observed to display high FR $\alpha$  in the primary tumor in contrast to low FR $\alpha$  in the paired metastatic tumor. A representative case with low FR $\alpha$  expression in the primary tumor and high FR $\alpha$  in the lymph node metastasis is shown in **Fig. 2C**.

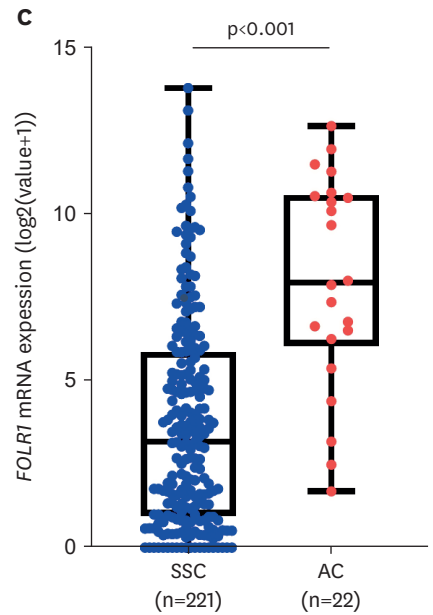
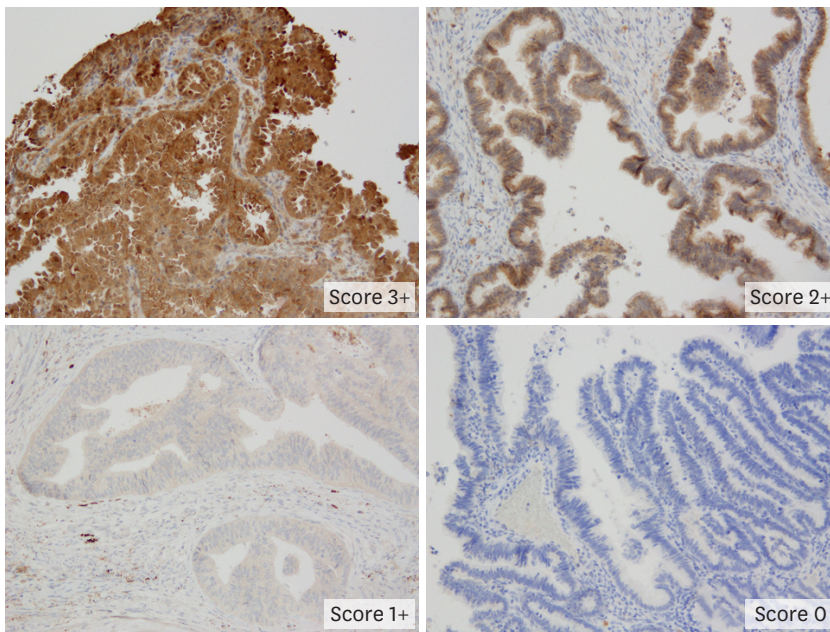
### 4. Association between high FR $\alpha$ expression and survival

We evaluated the association between FR $\alpha$  expression in primary tumors and survival. The median follow-up period was 62.6 months (95% confidence interval [CI]=9.1–128.9 months). There was a significant difference in DFS between the FR $\alpha$ -high and FR $\alpha$ -low groups (hazard ratio [HR]=2.05; 95% CI=1.08–3.89; log-rank  $p = 0.025$ ). The estimated 5-year DFS was 55.8%

**A** Squamous cell carcinoma ( $\times 200$ )



**B** Adenocarcinoma ( $\times 200$ )



**Fig. 1.** FR $\alpha$ -expression in different histological types of cervical cancer. Representative intensity patterns for FR $\alpha$  expression in (A) SCC and (B) AC. (C) Difference in *FOLR1* mRNA expression between SCC and AC using TCGA dataset. The dots depict the *FOLR1* expression in each patient. The box represents the first quartile, median, and third quartile; whiskers represent the minimum/maximum values. Red and blue dots refer to AC and SCC, respectively. AC, adenocarcinoma; FR $\alpha$ , folate receptor  $\alpha$ ; SCC, squamous cell carcinoma; TCGA, The Cancer Genome Atlas.

(95% CI=36.0%–71.7%) in patients with high FR $\alpha$  expression and 75.3% (95% CI=64.7%–83.2%) in those with low FR $\alpha$  expression (**Fig. 3A**). There was also a significant difference in OS between the FR $\alpha$ -high and FR $\alpha$ -low groups (HR=4.12; 95% CI=1.62–10.46; log-rank  $p=0.0013$ ). The estimated 5-year OS was 72.9% (95% CI=52.9%–85.4%) in patients with high FR $\alpha$  expression and 91.1% (95% CI=82.6%–95.7%) in patients with low FR $\alpha$  expression

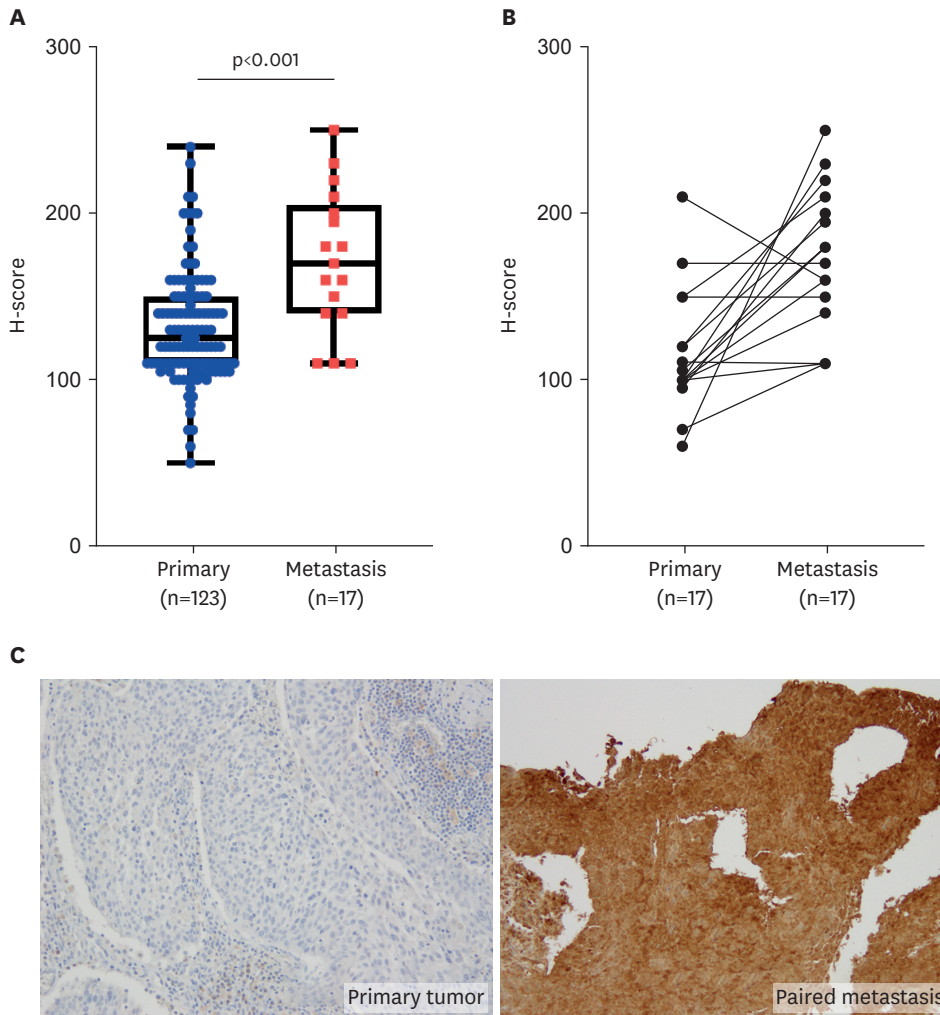
**FR $\alpha$  expression in cervical cancer**

**Table 2.** FR $\alpha$  expression in primary tumors by histological types of cervical cancer

Variables	Histological type					Total (n=123)
	SCC (n=67)	AC (n=27)	GAS (n=12)	ASC (n=13)	NEC (n=4)	
H-score	120 (110-140)	125 (105-160)	140 (132.5-167.5)	150 (110-162.5)	106 (63-117.5)	125 (110-150)
FR $\alpha$ -high	10 (14.9)	9 (33.3)	5 (41.7)	7 (53.8)	0 (0)	32 (26)
FR $\alpha$ -low	56 (83.6)	18 (66.6)	7 (58.3)	6 (56.2)	4 (100)	91 (74)

Values are presented as median (interquartile range) or number (%).

AC, adenocarcinoma; ASC, adenosquamous carcinoma; FR $\alpha$ , folate receptor  $\alpha$ ; GAS, gastric-type adenocarcinoma; NEC, neuroendocrine carcinoma; SCC, squamous cell carcinoma.



**Fig. 2.** FR $\alpha$ -expression in the primary and paired metastatic tumors.

(A) FR $\alpha$  expression in the total patient population. The dots depict the FR $\alpha$  expression in each patient. The box represents the first quartile, median, and third quartile; whiskers represent the minimum and maximum values. (B) FR $\alpha$  expression in primary and paired metastatic tumors. (C) Representative FR $\alpha$ -stained sections for primary and paired metastatic tumors.

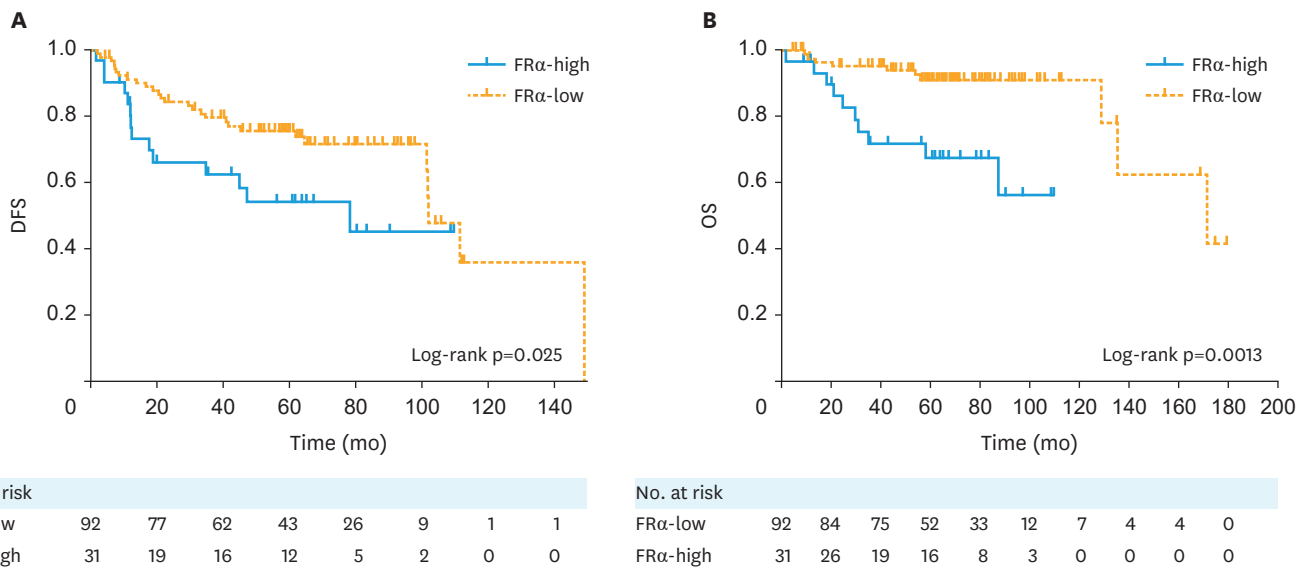
FR $\alpha$ , folate receptor  $\alpha$ .

(**Fig. 3B**). Multivariable analysis included age, histology, FIGO stage, lymph node metastasis, LVSI, adjuvant RT/CCRT, and FR $\alpha$  expression and showed that high FR $\alpha$  expression was significantly associated with worse DFS (HR=2.26; 95% CI=1.08–4.72; p=0.031; **Table S1**) and OS (HR=6.73; 95% CI=2.21–20.53; p=0.001; **Table 3**).

**Table 3.** Association between FR $\alpha$  expression and overall survival

Variables	Univariable			Multivariable		
	HR	95% CI	p-value	HR	95% CI	p-value
Age (continuous)	1.00	0.96–1.05	0.87	1.02	0.97–1.07	0.51
FIGO stage (I vs. II)	1.49	0.53–4.19	0.45	0.73	0.25–2.15	0.57
Lymph node metastasis (negative vs. positive)	10.51	3.05–36.24	<0.001	27.11	5.25–139.8	<0.001
Histological type (SCC vs. non-SCC)	1.27	0.53–3.08	0.59	0.84	0.31–2.26	0.73
LVSI (no vs. yes)	3.24	0.94–11.18	0.063	1.01	0.25–4.07	0.99
Adjuvant RT/CCRT (no vs. yes)	2.74	1.03–7.30	0.044	0.28	0.08–1.03	0.055
FR $\alpha$ expression (low vs. high)	4.12	1.62–10.46	0.003	6.73	2.21–20.53	0.001

CCRT, concurrent chemoradiotherapy; CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics; FR $\alpha$ , folate receptor  $\alpha$ ; HR, hazard ratio; LVSI, lymphovascular space involvement; RT, radiotherapy; SCC, squamous cell carcinoma.



**Fig. 3.** Kaplan-Meier analysis of DFS and OS according to FR $\alpha$ -high expression. (A) DFS and (B) OS in patients with high FR $\alpha$  expression vs patients with low FR $\alpha$  expression. DFS, disease-free survival; FR $\alpha$ , folate receptor  $\alpha$ ; OS, overall survival.

## DISCUSSION

To our knowledge, this is the first study to examine FR $\alpha$  expression in various histological types and prognostic outcomes in cervical cancer. High FR $\alpha$  expression was found in 25% of the primary tumors, was significantly more frequent in non-SCC histology than in SCC histology, and was an independent poor prognostic factor. In addition, FR $\alpha$  expression was higher in metastatic tumors than in the corresponding primary tumors.

FR $\alpha$  overexpression has been reported in 76%–89% of serous ovarian cancer, 35%–74% of TNBC, 20%–50% of endometrial cancer, and 14%–74% of NSCLC patients [25,26]. In our study, 25% of the patients with cervical cancer showed high FR $\alpha$  expression. FR $\alpha$  expression differed by histological type and was higher in non-SCC than in SCC (38% vs. 15%). Using the TCGA dataset, we further confirmed that *FOLR1* mRNA expression was higher in AC than in SCC. Previous studies in other cancer types have also shown that FR $\alpha$  expression varies according to histological type. A study of FR $\alpha$  expression in ovarian cancer revealed that FR $\alpha$  was expressed in 76% of high-grade serous carcinoma (HGSC) and 32% of clear cell carcinoma [27]. Furthermore, in a study involving NSCLC patients, FR $\alpha$  expression was found to be significantly higher in AC than in SCC [28]. Cervical adenocarcinoma has been increasing worldwide,



especially among young women [2,29], and GAS incidence is not expected to decrease even after widespread HPV vaccination since GAS is regarded as HPV-independent [21,30]. Patients with AC and GAS of the cervix are more resistant to standard treatments and have a poorer prognosis than those with SCC [31,32]. Therefore, new therapeutic strategies are needed for patients with AC and GAS of the cervix, and FR $\alpha$  may become an attractive therapeutic target.

This study showed that high FR $\alpha$  expression was an independent factor for poor prognosis. The prognostic impact of FR $\alpha$  expression has been evaluated in various cancer types. Although a recent meta-analysis indicated that high expression of FR $\alpha$  was associated with poor survival [33], its prognostic impact remains controversial. According to a study of 91 patients with HGSC, high FR $\alpha$  expression was a predictive marker of chemoresistance and was associated with poor prognosis [34]. On the other hand, an ovarian tumor tissue analysis consortium study of 1,400 patients with HGSC showed that FR $\alpha$  expression was not associated with progression-free survival and OS [27]. Differences in patient populations, staining, and scoring methods may have affected these results. Indeed, larger studies are needed to confirm the prognostic impact of FR $\alpha$  expression in cervical cancer.

FR $\alpha$  expression was higher in metastatic tumors than in the corresponding primary tumors. Of note, 61% of the patients with low FR $\alpha$  expression in the primary tumor exhibited high FR $\alpha$  expression in the metastatic tumor. Martin et al. compared FR $\alpha$  expression in archival and post-recurrence biopsy samples of ovarian cancer; this study revealed that in 2 out of 5 patients (40%), the archival tumor samples displayed low FR $\alpha$  expression, whereas the post-recurrence biopsy samples displayed high FR $\alpha$  expression [16]. Thus, more patients may benefit from FR $\alpha$ -targeted therapy through the re-biopsy of metastatic tumors.

This study had several limitations. First, it was a retrospective study, and a relatively small number of patients were included from a single institution. Our cohort included a relatively lower frequency of SCC (62%) than what is found in the general population. According to the population-based cancer registry in Japan, patients with SCC comprised 75.5% of cervical cancer patients in 2005 [35]. Furthermore, because of the long enrollment period, changes in treatment strategies may have affected the prognosis. Therefore, the generalizability of the prognostic impact of FR $\alpha$  expression remains uncertain. Second, we found increased FR $\alpha$  expression in the paired metastatic tumors, but the mechanism was unclear. One possibility is that clonal selection occurred during tumor progression, whereas postoperative treatment, site of recurrence, and time to relapse may also be associated. Since only 17 patients had paired metastatic samples, we were unable to evaluate the potential effects of these factors. Third, the association of HPV status and genomic alterations with FR $\alpha$  expression was not evaluated. In ovarian, breast, and endometrial cancers, FR $\alpha$  expression has been reported to be higher in HGSC, TNBC, and non-endometrioid (type II) cancers [27,36,37], in which most tumors are known to have *TP53* mutations. These results raised the hypothesis that genomic alterations, such as *TP53* mutations, may be associated with FR $\alpha$  expression. Our results support this hypothesis because the frequency of *TP53* mutation is higher in AC than in SCC in cervical cancer (AC vs. SCC, 20.9% vs. 5.6%) [38]. Further evaluation is required to confirm this hypothesis.

In conclusion, 25% of cervical cancers had high FR $\alpha$  expression, which was more prevalent in non-SCCs. High FR $\alpha$  expression was significantly associated with poor prognosis and was increased in metastatic tumors compared with primary tumors. FR $\alpha$  expression is a promising therapeutic target, and the development of FR $\alpha$ -targeted therapy may be desirable for the treatment of advanced cervical cancer.

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## SUPPLEMENTARY MATERIAL

### Table S1

Association between FR $\alpha$  expression and disease-free survival

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