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FOLR1 was up-regulated in cervical squamous cell carcinoma and correlated with the patients' progression free survival

<https://doi.org/10.1515/pteridines-2020-0014>

received January 20, 2020; accepted April 30, 2020.

Abstract: Objective The aim of the present work was to evaluate the folate-receptor 1 (*FOLR1*) expression in cervical squamous cell carcinoma and its clinical significance.

Methods *FOLR1* mRNA expression level was detected in the cancer genome atlas (TCGA) database for multiple carcinomas. The *FOLR1* mRNA relative expression between tumor tissue and normal cervix tissue of the cervical squamous cell cancer patients was compared by the online data analysis tool of GEPIA. The overall survival (OS) and progression free survival (PFS) between the *FOLR1* high and low expression groups were compared by the log-rank test. Thirty one cervical squamous cancer patients and 20 healthy controls were included in and tested for serum *FOLR1* protein level detection. Eighty one cervical squamous cell cancer patients who received surgery were included for *FOLR1* protein expression detected by immunohistochemistry assay (IHC). The correlation between *FOLR1* protein expression and patients' clinical features was analyzed.

Results *FOLR1* mRNA was up-regulated in tumor tissue compared to corresponding normal cervical tissue of cervical squamous cell carcinoma. Top 20 genes interacted with *FOLR1* was identified through the network with the edges of 146. *UBXN10* ($r=0.668$, $P<0.01$) and *GBP6* ($r=-0.606$, $P<0.01$) were the top 2 genes that most correlated with *FOLR1*. The serum level of FR- α (*FOLR1* coding protein) were 275.50 ± 83.79 and 161.70 ± 66.62 (ng/L)

for the cervical cancer and healthy control subjects respectively with significant statistical difference ($P<0.05$). Using the serum FR- α as serological marker for cervical cancer detection, the diagnostic sensitivity, specificity and AUC were 80.0% (58.40% to 91.93%), 80.65% (63.72% to 90.81%) and 0.85(95%CI:0.74-0.96), respectively. Immunohistochemical assay indicated that of the 81 cancer tissue samples, 45 (55.6%) was *FOLR1* protein positive. *FOLR1* protein positive expression rate in FIGO stage III/IV was significant higher than in the stage I/II with statistical difference ($P<0.05$). The progression free survival (PFS) was significant different between *FOLR1* high and low expression group (HR=2.48, 95%CI:1.1-5.58, $P=0.023$). However, the overall survival (OS) was not statistical different between the two groups (HR=1.34, 95%CI:0.84-2.15, $P=0.22$).

Conclusion: *FOLR1* was up-regulated in both serum and cancer tissue of cervical squamous cell carcinoma which may act as diagnostic and prognostic maker for cervical squamous cell cancer.

Keywords: *FOLR1*; cervical squamous cell carcinoma; bioinformatics analysis; survival; immunohistochemistry.

Introduction

Cervical cancer is one of the most diagnosed female productive system malignant carcinoma [1]. It was conformed that the development of cervical cancer was related to high-risk human papillomavirus (HPV) infection [2, 3]. Multiple sexual partners is the independent high risk factor of HPV infection [4]. In recent years, the epidemiological data showed that the incidence of cervical cancer had a significant upward trend, especially in youngers [5-7]. The prognosis of patients with early stage cervical cancer was good with relative high 5-year survival rate, but the prognosis of patients with advanced cervical cancer was poor. However, the carcinogenesis

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and prognosis of cervical cancer was still not clear yet. In recent years, with the development of molecular biology technology, more and more studies have confirmed that the abnormal expression of some genes was of great significance in the carcinogenesis, development and prognosis of cervical cancer.

Folate receptor-1 (FOLR1/FR- α), a glycosylphosphatidyl alcohol coupled glycoprotein, has a molecular weight of $38\text{--}40 \times 10^3$ and is encoded by FOLR1 gene. FOLR1 plays an important role in DNA synthesis, cell metabolism, proliferation and division. Liu et al. [8] reported that FOLR1/FR- α protein may affect the growth of cervical cancer cells by activating ERK/c-fos/c-Jun signaling pathway. Bai et al. [9] found that the down-regulation of FOLR1 expression inhibited the proliferation and increased the apoptosis rate of cervical cancer cells, suggesting that FOLR1 played a role in the proliferation and apoptosis of cervical cancer cells. However, whether FOLR1 expression in cervical squamous cell carcinoma and its role as biomarker for diagnosis and prognosis of cervical cancer is unclear.

Material and methods

Bioinformatics analysis

FOLR1 mRNA expression level was detected in the cancer genome atlas (TCGA) database for multiple carcinomas. The FOLR1 mRNA relative expression between tumor tissue and normal cervix tissue of the cervical squamous cell cancer patients were compared by the online data analysis tool of GEPIA [10]. Protein-protein interaction (PPI) network relevant FOLR1 gene was constructed through the STRING database [11] to evaluate the interaction proteins relevant to FOLR1 with the minimum required interaction score no less than 0.4. The top 50 positive and negative co-expression genes with FOLR1 were also identified and made cluster analysis and demonstrated by the heat map. The top 1 positive and negative correlated genes with FOLR1 were further analyzed by Pearson correlation test. The cervical cancer patients were divided into two high expression ($>$ median FOLR1 mRNA) and low expression ($\leq$ median FOLR1 mRNA) groups. The overall survival and progression free survival between the high and low expression groups were compared by the log-rank test.

Enzyme linked immunosorbent assay

Thirty one cervical squamous cancer patients and 20 healthy controls were included in for serum FOLR1 protein level detection. The general characteristic of the included cervical cancer and healthy controls was demonstrated in **Table 1**. 3 ml of elbow vein blood was extracted and centrifugation for 5 minutes with the speed of 2500 r/min. Then the upper serum was collected and stored in a refrigerator at -70°C . Enzyme linked immunosorbent assay (ELISA) was used to detect the serum expression level of FOLR1.

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the ethical committee of Ji'nan Zhangqiu District Maternal and Child Health Hospital.

Informed consent: Informed consent has been obtained from all individuals included in this study

Immunohistochemistry assay

Eighty one cervical squamous cell cancer patients who received surgery were included for FOLR1 protein expression detection by immunohistochemistry assay (IHC). The paraffin samples of cervical cancer were excised and sectioned continuously with the thickness $4\text{ }\mu\text{m}$. The slices were fixed with 95% ethanol for 10 min, washed with distilled water twice for 15 s each time, washed with PBS solution three times for 15 s each time, mixed with anhydrous ethanol and 3% H_2O_2 (1:9) and dropped for 5 min with 10% volume 0%, 90% and 80% ethanol gradient hydration. PBS solution washing twice, 15 s each time; add 40 μl rabbit anti human FR- α protein I antibody (1:100) incubate for 1 h at 37°C ; PBS solution washing three times, 15 s each time; drop DAB solution 200 $\sim$ 300 μl , water bath at room temperature for 2 min, then immediately put it into deionized water after observing the color under microscope, dye hematoxylin again for 1 min, differentiate, dehydrate with 100% ethanol volume fraction after the slice is returned to blue, soak it in xylene until transparent, and seal it with neutral gum. The positive control was confirmed cervical cancer tissue section or cervical intraepithelial neoplasia section, and the negative control was PBS solution.

Table 1: General characteristics of the included cervical cancer and healthy controls.

Features	Cervical cancer (n=31)	Control (n=20)	t/ χ^2	P
Age (year)			0.63	0.43
<=50	12	10		
>50	19	10		
FR- α (ng/L)	275.50 $\pm$ 83.79	161.70 $\pm$ 66.62		
HPV(16)			39.35	<0.001
Negative	2	19		
Positive	29	1		
Gravidity			0.06	0.81
<=2	15	9		
>2	16	11		
Artificial abortion			0.20	0.65
Negative	9	7		
Positive	22	13		
Family tumor history			1.34	0.25
Negative	29	20		
Positive	2	0		

Statistical methods

Stata12.0 statistical software was applied for data analysis. Measurement data was expressed by $\bar{x} \pm s$, and made student-t test. Counting data was expressed by number and made chi-square test. $P < 0.05$ was considered as statistically significant.

Results

FOLR1 expression detected in TCGA database

FOLR1 mRNA expression in cervical squamous cell cancer and other malignant tumors were demonstrated in **Figure 1**. FOLR1 mRNA expression level was quite different among different carcinomas (**Figure 1A**). For cervical cancer patients, FOLR1 mRNA was up-regulated in tumor tissue compared to corresponding normal cervical tissue (**Figure 1B**). And FOLR1 mRNA level was correlated with the patients clinical stages ($P < 0.05$), **Figure 1C**.

PPI network of FOLR1

The protein-protein interaction network relevant to FOLR1 and related protein coding genes were constructed by the STRING database. The top 20 genes interaction with FOLR1 was identified through the network with the edges of 146, **Figure 2**.

FOLR1 co-expression gene analysis

The top 50 positive and negative correlated genes with FOLR1 were demonstrated by the heat map (**Figure 3**). UBXL10($r=0.668$, $P < 0.01$) and GBP6($r=0.606$, $P < 0.01$) were the top 2 genes that most correlated with FOLR1, **Figure 4**.

Serum FOLR1 protein (FR- α) level

The serum level of FR- α were 275.50 $\pm$ 83.79 and 161.70 $\pm$ 66.62 (ng/L) for the cervical cancer and healthy control subjects respectively with significant statistical difference ($P < 0.05$), **Figure 5A**.

Figure 3: Heat map of FOLR1 and correlated genes (A: Heat map of FOLR1 positive correlated genes; B: Heat map of FOLR1 negative correlated genes).

Figure 4: Scatter plot of FOLR1 positive and negative correlated genes(A: UBXL10 was positive correlated with FOLR1; B: GBP6 was negative correlated with FOLR1).

Diagnostic efficacy by using the serum FR-α

Using the serum FR-α as serological marker for cervical cancer detection, the diagnostic sensitivity, specificity and AUC were 80.0% (58.40% to 91.93%), 80.65% (63.72% to 90.81%) and 0.85(95%CI: 0.74-0.96) , respectively, **Figure 5B**.

FOLR1 protein expression detected by immunohistochemistry assay

Immunohistochemical assay indicated that the FOLR1 protein was positively expressed in the nucleus with yellow brown staining (**Figure 6**). And in the 81 cancer tissue samples, 45 (55.6%) was FOLR1 protein positive.

FOLR1 protein expression and patients clinical characteristics

FOLR1 protein positive expression rate in FIGO stage III/IV was significant higher than in the I/II with statistical difference ($P<0.05$). However, FOLR1 protein positive expression was not associated with the patients' age, tumor diameter, lymph node metastasis and tumor differentiation ($P_{all}>0.05$), **Table 2**.

FOLR1 expression and patients' survival

The progression free survival (PFS) was significant different between FOLR1 high and low expression group (HR=2.48, 95%CI:1.1-5.58, $P=0.023$). However, the overall

Figure 5: Diagnostic efficacy of serum FOLR1 protein as biomarker for cervical cancer(A: Scatter plot of serum FR-α distribution between cervical cancer and healthy controls; B: ROC curve of serum FR-α as biomarker for cervical squamous cell carcinoma diagnosis).

Figure 6: Immunohistochemistry assay in detection FOLR1 protein expression in cervical squamous cell carcinoma and normal cervical tissues.

survival (OS) was not statistical different between the two groups (HR=1.34, 95%CI:0.84-2.15, P=0.22), **Figure 7**.

Discussion

According to genomics analysis, most malignant tumors have gene mutations or changes in the expression level, suggesting that genomic instability or abnormal expression plays an important role in the carcinogenesis and development of tumors [12, 13]. Cervical cancer is a common gynecological malignant tumor, which is related to high-risk human papillomavirus(HPV) infection. In recent years, it has been reported that the differential expression of oncogenes and tumor suppressor genes played an important role in the occurrence and

development of cervical cancer, and had correlation with the patients' prognosis [14].

Folate is the main component of S-adenosylmethionine, and S-adenosylmethionine is the provider of maintaining the normal metabolism of methyl in the human body. It has been confirmed that appropriate supplement of folate can reverse precancerous lesions, suggesting that folate plays an important role in cell proliferation, division and tissue growth [15]. It was found that cervical cancer was a chronic process caused by many factors such as HPV persistent infection. Studies about correlation between HPV infection and cervical cancer susceptibility have shown that the lack of folate in tumor cells can reduce the methylation level and lead to the carcinogenesis of cervical cancer [16]. FOLR1 is a membrane protein, which can be coupled with GPI and can internalize and mediate

Figure 7: Survival curve of cervical cancer between FOLR1 mRNA high and low expression groups (A: overall survival; B: progression free survival).

Table 2: FOLR1 protein expression and cervical squamous cell carcinoma clinical pathological characteristics.

Features	n=81	FOLR1 expression		χ^2	P
		Positive(n=45)	Negative(n=36)		
Age (year)				0.49	0.48
<=50	37	19	18		
>50	44	26	18		
FIGO stage				5.63	0.02
I/II	54	25	29		
III/IV	27	20	7		
Tumor diameter(cm)				1.47	0.22
<=4	48	24	24		
>4	33	21	12		
Lymph node metastasis				1.05	0.31
Negative	50	30	20		
Positive	31	15	16		
Differentiation				0.06	0.80
Well/moderate	44	25	19		
Poor	37	20	17		

folate into cells. It has a high affinity for folate and its derivatives [17, 18]. It has been demonstrated that FOLR1 was up-regulated in many malignant tumors because of the increased metabolic demand of folate caused by the rapid growth of tumor cells, indicating that FOLR1 plays

an important role in cell metabolism, repair of synthetic DNA, proliferation and division of tumor cells [19].

In our present study, we investigate the expression level of FOLR1 gene in a variety of human tumors through bioinformatics analysis, and compared the different

expression of FOLR1 gene mRNA in cervical cancer tissues and adjacent normal tissues. The results showed that the expression level of FOLR1 was different among different human tumors. The expression level of FOLR1 mRNA in cervical cancer was significantly higher than that in normal cervical epithelium, suggesting that FOLR1 may play an important role in the development of cervical cancer. Survival analysis showed that the PFS of patients with high expression of FOLR1 gene was lower than that of patients with low expression ($P < 0.05$). The survival data suggested that the high expression of FOLR1 was a risk factor for the short survival and poor prognosis of cervical cancer patients. Therefore, FOLR1 expression in patients with cervical cancer can be used as a molecular marker of prognosis. We also detected the expression level of FOLR1 protein in the serum of cervical cancer patients and healthy controls. The results indicated the FOLR1 protein in the serum of cervical cancer patients was significantly higher than that in the healthy control group, and it could be used as a diagnostic serological marker of cervical cancer. However, the sample size of our own data set was small and the statistical power was limited which need further validation.

We also performed the IHC assay to detect the expression of FOLR1 protein in 81 cervical cancer patients and evaluated its relationship with the patients' clinicopathological characteristics. The high expression rate of FOLR1 protein in cervical cancer was 55.6% (45 / 81). The high expression of FOLR1 protein was related to FIGO stage ($P < 0.05$). FIGO stage and lymph node metastasis were the prognostic factors of cervical cancer patients. The late stage of high expression of FOLR1 validated the relationship between high expression of FOLR1 and poor prognosis.

In conclusion, the expression of FOLR1 gene was increased in cervical cancer tissues, which may be a molecular marker of diagnosis and prognosis of cervical cancer patients.

Conflict of interest: Authors state no conflict of interest.

Data Availability Statement: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin.* 2019 Jan;69(1):7–34.
2. Findik S, Findik S, Abuoğlu S, Cihan FG, Ilter H, Iyisoy MS. Human papillomavirus (HPV) subtypes and their relationships with cervical smear results in cervical cancer screening: a community-based study from the central Anatolia region of Turkey. *Int J Clin Exp Pathol.* 2019 Apr;12(4):1391–8.
3. Torres-Poveda K, Ruiz-Fraga I, Madrid-Marina V, Chavez M, Richardson V. High risk HPV infection prevalence and associated cofactors: a population-based study in female ISSSTE beneficiaries attending the HPV screening and early detection of cervical cancer program. *BMC Cancer.* 2019 Dec;19(1):1205.
4. Liu ZC, Liu WD, Liu YH, Ye XH, Chen SD. Multiple Sexual Partners as a Potential Independent Risk Factor for Cervical Cancer: a Meta-analysis of Epidemiological Studies. *Asian Pac J Cancer Prev.* 2015;16(9):3893–900.
5. Chan CK, Aimagambetova G, Ukybassova T, Kongrtay K, Azizan A. Human Papillomavirus Infection and Cervical Cancer: Epidemiology, Screening, and Vaccination-Review of Current Perspectives. *J Oncol.* 2019 Oct;2019:3257939.
6. Pedersen K, Fogelberg S, Thamsborg LH, Clements M, Nygård M, Kristiansen IS, et al. An overview of cervical cancer epidemiology and prevention in Scandinavia. *Acta Obstet Gynecol Scand.* 2018 Jul;97(7):795–807.
7. Hillemanns P, Soergel P, Hertel H, Jentschke M. Epidemiology and Early Detection of Cervical Cancer. *Oncol Res Treat.* 2016;39(9):501–6.
8. Liu C, Ding L, Bai L, Chen X, Kang H, Hou L, et al. Folate receptor alpha is associated with cervical carcinogenesis and regulates cervical cancer cells growth by activating ERK1/2/c-Fos/c-Jun. *Biochem Biophys Res Commun.* 2017 Sep;491(4):1083–91.
9. Bai LX, Ding L, Jiang SW, Kang HJ, Gao CF, Chen C, et al. Down-regulation of FR α inhibits proliferation and promotes apoptosis of cervical cancer cells in vitro. *Asian Pac J Cancer Prev.* 2014;15(14):5667–72.
10. Tang Z, Li C, Kang B, Gao G, Li C, Zhang Z. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. *Nucleic Acids Res.* 2017 Jul;45 W1:W98–102.
11. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* 2019 Jan;47 D1:D607–13.
12. Iliakis G, Mladenov E, Mladenova V. Necessities in the Processing of DNA Double Strand Breaks and Their Effects on Genomic Instability and Cancer. *Cancers (Basel).* 2019 Oct;11(11):11.
13. Persi E, Prandi D, Wolf YI, Pozniak Y, Barnabas GD, Levanon K, et al. Proteomic and genomic signatures of repeat instability in cancer and adjacent normal tissues. *Proc Natl Acad Sci USA.* 2019 Aug;116(34):16987–96.
14. Massad LS. Methods of Screening for Cervical Cancer-Reply. *JAMA.* 2018 Nov;320(18):1939.
15. Pachón H. [Folate and breast cancer risk]. *Rev Med Chil.* 2013 Feb;141(2):272.

16. Akhtar A, Ghali L, Wang SX, Bell C, Li D, Wen X. Optimisation of Folate-Mediated Liposomal Encapsulated Arsenic Trioxide for Treating HPV-Positive Cervical Cancer Cells In Vitro. *Int J Mol Sci.* 2019 Apr;20(9):20.
17. da Costa M, Rothenberg SP, Sadasivan E, Regec A, Qian L. Folate deficiency reduces the GPI-anchored folate-binding protein in rat renal tubules. *Am J Physiol Cell Physiol.* 2000 Apr;278(4):C812–21.
18. Wang X, Jansen G, Fan J, Kohler WJ, Ross JF, Schornagel J, et al. Variant GPI structure in relation to membrane-associated functions of a murine folate receptor. *Biochemistry.* 1996 Dec;35(50):16305–12.
19. Leung F, Dimitromanolakis A, Kobayashi H, Diamandis EP, Kulasingam V. Folate-receptor 1 (FOLR1) protein is elevated in the serum of ovarian cancer patients. *Clin Biochem.* 2013 Oct;46(15):1462–8.