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Long noncoding RNA PVT1 as a novel serum biomarker for detection of cervical cancer

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Abstract. – OBJECTIVE: To investigate long noncoding RNA PVT1 expression in the serum of cervical cancer patients, and to evaluate serum PVT1 level as a diagnostic biomarker for cervical cancer.

PATIENTS AND METHODS: Eighty-eight cervical cancer patients, 64 cervical intraepithelial neoplasia patients, 25 breast cancer patients, 25 ovarian cancer patients, and 111 healthy control subjects were enrolled into this study. PVT1 serum level in these participants and PVT1 expression in 20 pairs of cervical cancer tissues and adjacent paired normal tissues was measured by quantitative reverse transcription-polymerase chain reaction. The diagnostic values of serum PVT1 were evaluated by receiver operating characteristic curves analysis.

RESULTS: Serum PVT1 level is significantly increased in cervical cancer patients and correlated with tumor size, clinical stage, and lymph node metastasis of cervical cancer. Serum PVT1 could accurately discriminate cervical cancer patients from cervical intraepithelial neoplasia patients and healthy control subjects, and also discriminate early stage cervical cancer patients from healthy control subjects. But serum PVT1 level is not changed in breast cancer and ovarian cancer patients. Furthermore, serum PVT1 level is positively correlated with tissue PVT1 expression, and could indicate cervical cancer dynamics.

CONCLUSIONS: Long noncoding RNA PVT1 may be a novel noninvasive biomarker for early diagnosis of cervical cancer.

Key Words:
Long noncoding RNA, PVT1, Cervical cancer, Serum, Biomarker, Diagnosis.

Introduction

Cervical cancer (CC) is the fourth most common cancer and fourth most frequent cause of cancer mortality affecting woman in the world[1]. There were 527,600 estimated new cases of CC and 265,700 estimated deaths of CC in 2012 worldwide[1]. The prognosis of CC patients is closely associated with the clinical stage[2]. Most of CC patients with early stage could be successfully cured, but the survival rate of CC patients diagnosed at the late stages is as low as 15%[3]. Therefore, early diagnosis and therapy could significantly reduce mortality of CC patients[4,5].

Although cervical smear cytological examination is the mainly screening method of early diagnosis of CC, the inconveniences of examining and high false-negative rate limit its application[6,7]. In addition to cervical smear cytological examination, serum biomarkers, including carbohydrate antigen 125 (CA125) and squamous cell carcinoma antigen (SCC Ag), are commonly used for the diagnosis of CC[8,9]. However, the low diagnostic sensitivity and specificity limit their utility[10]. Therefore, novel noninvasive diagnostic biomarkers with high sensitivity and specificity are urgently required.

Long noncoding RNAs (lncRNAs), which are longer than 200 nucleotides with limited protein-coding potential, have multiple functions in various pathophysiological processes and are dysregulated in many kinds of diseases, particular in cancers[11-15]. LncRNA PVT1, CCAT2, HOTAIR, XLOC_010588, MALAT1, lnc-CC3, and et al. have been reported to be upregulated in CC and promote CC progression[16-22]. While other lncRNAs, such as GAS5, LET, and MEG3 are downregulated in CC and inhibit CC progression[23-25]. However, whether these lncRNAs are present in peripheral blood and could be used as diagnostic biomarkers for CC is still unknown. LncRNAs are really detectable and could be used as diagnostic biomarkers for many cancers, such as lncRNA H19, CUDR, LSINCT-5, PTPEN1, and UCA1 for gastric cancer[26-28]; HOTTIP-005 and HOTTIP-001 for pancreatic cancer[29]; POU3F3

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for esophageal squamous cell carcinoma[30]; XIST and HIF1A-AS1 for non-small cell lung cancer[31]; UCA1, WRAP53, uc003wbd, and AF085935 for hepatocellular carcinoma[32,33].

Many reports have shown that lncRNA PVT1 was increased in CC tissues, associated with poor prognosis, and promoted CC development[16,17]. In this study, we further measured serum PVT1 levels in a large cohort of CC patients, cervical intraepithelial neoplasia (CIN) patients, and healthy control (HC) subjects, and analyzed the diagnostic sensitivity and specificity of serum PVT1 for CC. Our results showed that serum PVT1 is significantly increased in CC patients and could accurately discriminate CC patients from CIN patients and HC subjects. However, serum PVT1 is not changed in breast cancer and ovarian cancer patients. Furthermore, serum PVT1 level is positively correlated with tissue PVT1 expression, and serum PVT1 level is significantly decreased in post-surgery CC patients. Collectively, our data suggested that PVT1 may be a novel noninvasive diagnostic biomarker for CC.

Materials and Methods

Patients and Samples

The Ethics Committee of the Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine approved the collection and usage of human specimens. A total of 88 CC patients, 64 CIN patients, 25 breast cancer patients, 25 ovarian cancer patients, and 111 healthy control (HC) subjects were enrolled into this study at the Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine. All the patients were diagnosed and confirmed by pathological examination in our hospital. CC clinical stages were determined following the International Federation of Gynecology and Obstetrics (FIGO). All the HC subjects enrolled in this study were age-matched women with normal cervix examined in our hospital. Women diagnosed with other tumors, pregnancy, or other chronic diseases are excluded from our study. Twenty fresh CC tissues and adjacent paired normal tissues were obtained during the surgical resection in our hospital. None of the patients received radiation therapy, chemotherapy, or immunotherapy before the collection of peripheral blood or surgical resection. All the patients and HC subjects signed informed consent.

RNA Extraction

Venus blood was collected from fasting subjects and centrifuged at 3000 rpm for 15 min at 4 °C. The supernatant was immediately collected and stored at -80 °C until use. All CC tissues and paired normal tissues were immediately frozen in liquid nitrogen and stored at -80 °C until use after surgery. Total RNAs were extracted from tissues and serum using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

LncRNAs expressions in serum and tissues of CC patients were measured with qRT-PCR assays. Complementary DNA was synthesized using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) following the manufacturer's instructions. Quantitative PCR was performed using the SYBR® Premix Ex Taq™ II (Takara) on StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions in triplicate. PVT1 expression in serum and tissues was normalized to GAPDH using the comparative Ct method. The primer sequences used were as follows: for PVT1, 5'-ATAGATCCTGCCCTGTTTGC-3' (forward) and 5'-CATTTCCTGCTGCCGTTTTC-3' (reverse); for GAPDH, 5'-GGAGCGAGATCCCTCCAAAAT-3' (forward) and 5'-GGCTGTTGTCATACTTCTCATGG-3' (reverse).

Statistical Analysis

Statistical analyses were performed with the GraphPad Prism Software. Mann-Whitney U test or Wilcoxon signed-rank test was used to compare serum or tissues PVT1 level between different groups as indicated. Receiver operating characteristic (ROC) curves analysis was used to assess the sensitivity and specificity of serum PVT1 for CC diagnosis. Pearson correlation analysis was used to evaluate the correlation between serum and tissues PVT1 level. p-values < 0.05 was defined as statistically significant.

Results

PVT1 is Increased in the Serum of CC Patients

To evaluate the clinical significance of serum PVT1, we first quantified PVT1 expression by

qRT-PCR in the serum of 88 CC patients, 64 CIN patients, and 86 age-matched healthy control (HC) subjects. The clinical features of CC patients, CIN patients, and HC subjects are shown in Table I. In comparison to HC subjects, PVT1 was significantly increased in CIN patients, and was further increased in CC patients (Figure 1A). Next, we detected the correlation between serum PVT1 level and clinical features in the 88 CC patients. As shown in Figure 1B, PVT1 was increased in CC patients with larger tumor size. Furthermore, PVT1 was increased in CC patients with advanced FIGO stage (Figure 1C). In comparison to CC patients without lymph node metastasis, PVT1 was increased in CC patients with lymph node metastasis (Figure 1D).

Circulating PVT1 Could be Used as a Novel Noninvasive Biomarker for CC

To evaluate whether serum PVT1 could be used as a novel diagnostic biomarker for CC, re-

Table I. Clinical features of the cervical cancer (CC) patients, cervical intraepithelial neoplasia (CIN) patients and healthy control (HC) subjects.

Variable	CC	CIN	HC
All cases	88	64	86
Age			
< 50	49	36	45
≥ 50	39	28	41
Tumor size (cm)			
< 4	48		
≥ 4	40		
Histology			
Squamous	73		
Adenocarcinoma	15		
FIGO stage			
I	45		
II	39		
III	4		
Differentiation			
Well/moderate	58		
Poor	30		
Lymph node metastasis			
Negative	66		
Positive	22		

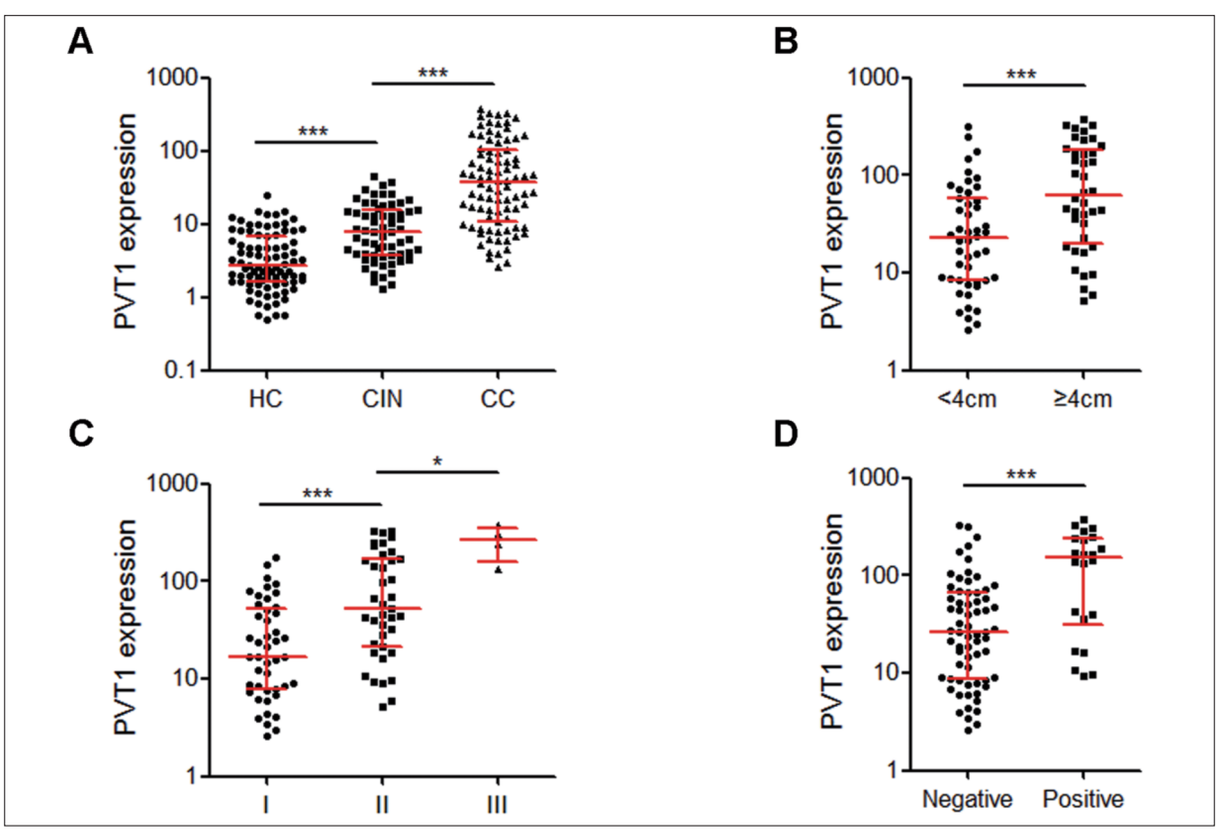

Figure 1. Serum PVT1 levels in CC and CIN patients and its correlation with clinical features. ***A,*** Serum PVT1 levels in 88 CC patients, 64 CIN patients, and 86 HC subjects. ***B,*** Serum PVT1 levels in CC patients with tumor size < 4 cm or tumor size ≥ 4 cm. ***C,*** Serum PVT1 levels in CC patients at different FIGO stages. ***D,*** Serum PVT1 levels in CC patients with or without lymph node metastasis. Serum PVT1 levels were measured by qRT-PCR. Results are presented as median with interquartile range. *$p < 0.05$, ***$p < 0.001$ by Mann-Whitney U test.

ceiver operating characteristic (ROC) curve analysis were performed. ROC curve showed accurate discrimination between CC patients and HC subjects, with an area under the ROC curve (AUC) of 0.932 (95% CI: 0.898-0.966), a sensitivity of 71.6%, and a specificity of 98.8% (Figure 2A). ROC curve also showed that serum PVT1 was sensitive and specific to discriminate CC patients from CIN patients, with an area under the ROC curve (AUC) of 0.817 (95% CI: 0.752-0.882), a sensitivity of 61.4%, and a specificity of 89.1% (Figure 2B), as well as CIN patients from HC subjects, with an area under the ROC curve (AUC) of 0.758 (95% CI: 0.683-0.834), a sensitivity of 87.5%, and a specificity of 52.3% (Figure 2C).

We next evaluated the clinical values of serum PVT1 in discriminating early stage CC patients from HC subjects. ROC curve showed good diagnostic sensitivity and specificity for stage I CC patients (AUC: 0.892; 95% CI: 0.838-0.946; sensitivity: 86.7%; specificity: 73.3%), and stage I & II CC patients as well (AUC: 0.929; 95% CI: 0.894-0.964; sensitivity: 71.4%; specificity: 97.7%) (Figure 2D and E). These data suggested that PVT1 could be used as a novel noninvasive biomarker for CC early diagnosis.

Diagnostic Specificity for CC of PVT1

To evaluate the diagnostic specificity of PVT1, we further measured serum PVT1 level in another cohort, including 25 HC subjects, 25 breast cancer (BC) patients, and 25 ovarian cancer (OC) patients. As shown in Figure 3, compared to HC subjects, serum PVT1 level was not significantly different in BC and OC patients. These results implied that PVT1 might be a specific diagnostic biomarker for CC.

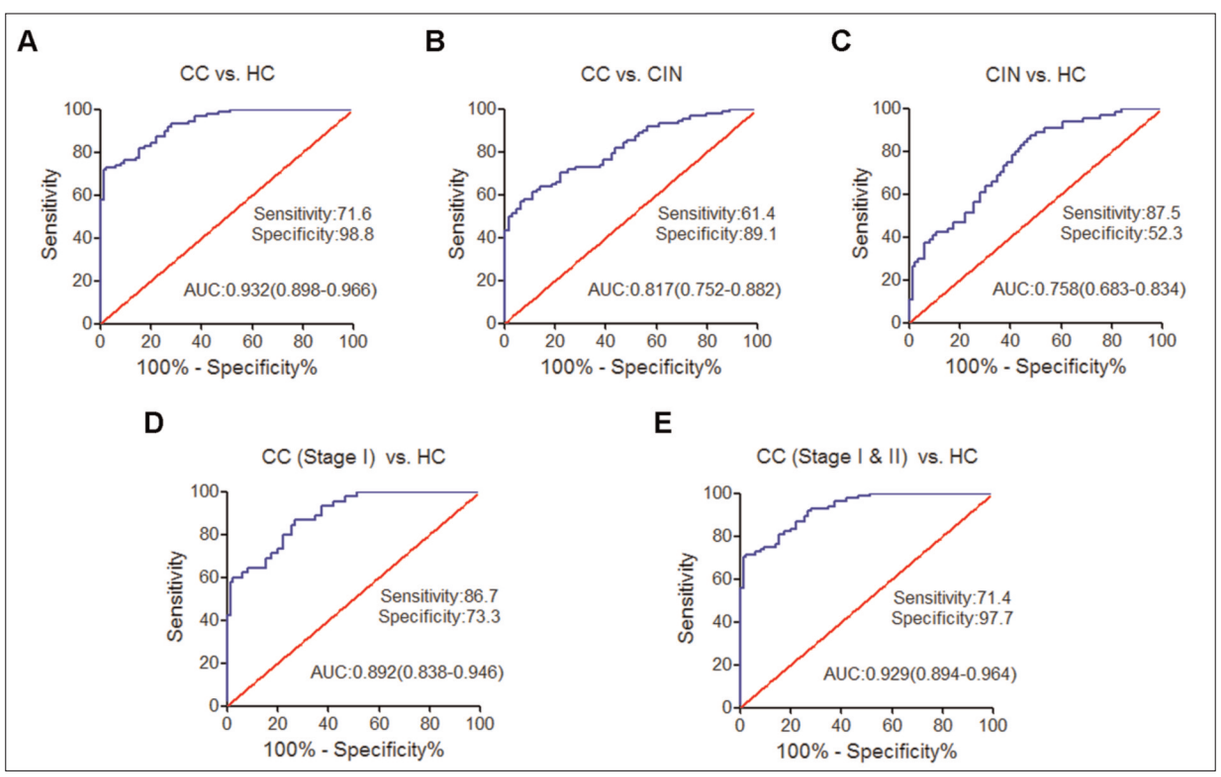

Figure 2. Diagnostic value of PVT1 for CC. ***A,*** ROC curve analysis of PVT1 for discrimination between CC and healthy control subjects (AUC: 0.932 (95% CI: 0.898-0.966), sensitivity: 71.6%, specificity: 98.8%). ***B,*** ROC curve analysis of PVT1 for discrimination between CC and CIN patients (AUC: 0.817 (95% CI: 0.752-0.882), sensitivity: 61.4%, specificity: 89.1%). ***C,*** ROC curve analysis of PVT1 for discrimination between CC and CIN patients (AUC: 0.758 (95% CI: 0.683-0.834), sensitivity: 87.5%, specificity: 52.3%). ***D,*** ROC curve analysis of PVT1 for discrimination between stage I CIN patients and HC subjects (AUC: 0.892 (95% CI: 0.838-0.946), sensitivity: 86.7%, specificity: 73.3%). ***E,*** ROC curve analysis of PVT1 for discrimination between stage I and II CC and CIN patients (AUC: 0.929 (95% CI: 0.894-0.964), sensitivity: 71.4%, specificity: 97.7%). $p < 0.001$ for all panels.

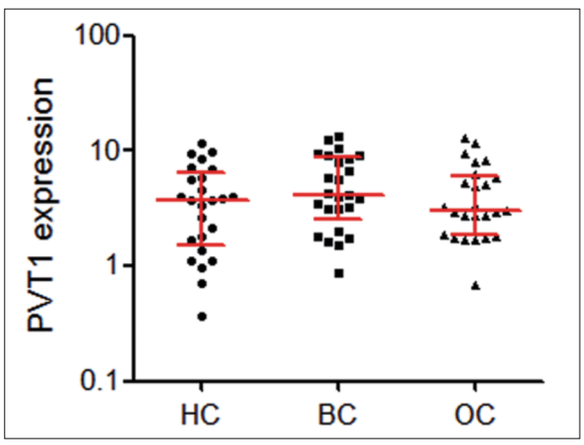

Figure 3. Serum PVT1 levels in breast cancer (BC) and ovarian cancer (OC) patients. Expression profiles of PVT1 in the serum of 25 HC subjects, 25 BC patients, and 25 OC patients. Serum PVT1 levels were measured by qRT-PCR. No statistical difference was observed between these three groups by Mann-Whitney U test.

Serum PVT1 Level Could be Used for Monitoring CC Dynamics

To further investigate the clinical values of serum PVT1 in monitoring CC dynamics, we measured PVT1 expression in 20 pairs of CC tissues and adjacent normal tissues using qRT-PCR. Consistent with PVT1 expression in serum of CC patients, PVT1 was also significantly increased in CC tissues compared with adjacent normal tissues (Figure 4A). A significant positive correlation was observed between serum PVT1 level of CC patients and PVT1 expression in CC tissues ($r = 0.838$, $p < 0.001$) (Figure 4B). Furthermore, we measured serum PVT1 level from 20 pairs of pre- and post-surgery CC patients who underwent radical resection. As shown in Figure 4C, serum PVT1 level was significantly decreased in post-surgery CC patients ($p < 0.001$). These data suggested that serum PVT1 level may indicate the expression of PVT1 in CC tissues and that PVT1 could be used for monitoring CC dynamics.

Discussion

Despite great progressions in CC diagnosis and therapy in the last twenty years, CC remains to have high mortality due to the late diagnosis[34]. Therefore it is urgent to develop novel noninvasive biomarkers for the early diagnosis of CC. Recently, many circulating nucleic acids have been investigated for their diagnostic values of cancers[35]. Serum microRNAs panel including miR-16-2*, miR-195, miR-2861, and miR-497, has been reported to be used for the early detection of CC[36]. In addition to microRNAs, lncRNAs, another important type of noncoding RNAs, have been reported to be detectable and used as potential serum biomarkers for early diagnosis of several cancers[32]. Although several microRNAs have been investigated in the early diagnosis of CC, the diagnostic value of lncRNAs in CC is still unknown.

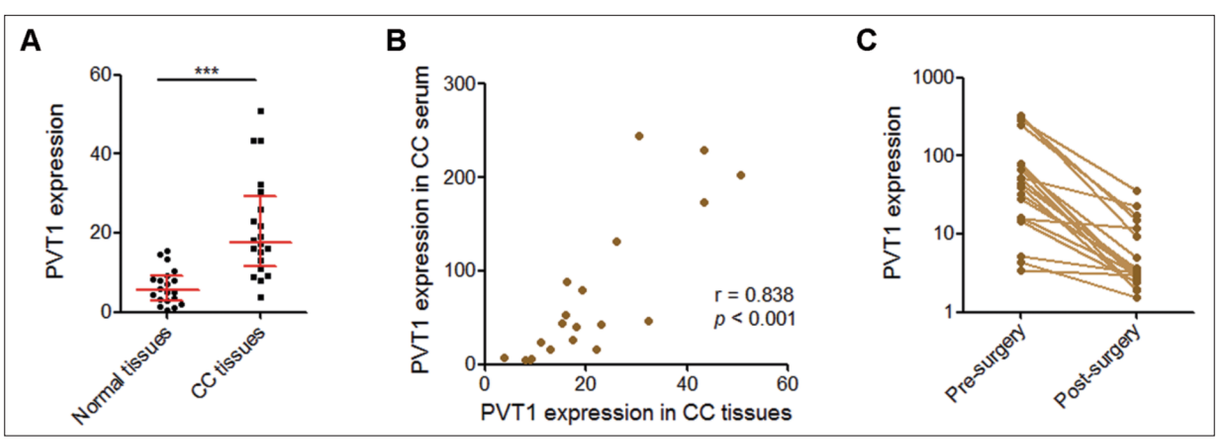

Figure 4. Serum PVT1 levels indicate CC dynamics. ***A,*** PVT1 expressions in 20 pairs of CC tissues and adjacent normal tissues were measured by qRT-PCR. Results are presented as median with interquartile range. ***$p < 0.001$ by Wilcoxon signed-rank test. ***B,*** Serum PVT1 level was significantly positive correlated with PVT1 expression in CC tissues. $r = 0.838$, $p < 0.001$ by Pearson correlation analysis. ***C,*** Serum PVT1 levels in 20 pairs of pre- and post-surgery CC patients were measured by qRT-PCR. $p < 0.001$ by Wilcoxon signed-rank test.

In this work, we focused on the lncRNA PVT1, which has been reported to be upregulated in CC tissues and function as an oncogene in CC[16, 17]. We measured serum PVT1 level in CC patients, CIN patients, and healthy control (HC) subjects. The results revealed that compared to HC subjects, serum PVT1 level is increased in CIN patients, and is further increased in CC patients. Serum PVT1 level in CC is correlated with tumor size, FIGO stage, and lymph node metastasis. ROC curve analysis showed that serum PVT1 level accurately discriminates CC patients from CIN patients and HC subjects, and also discriminates CIN patients from HC subjects. Furthermore, serum PVT1 level could also accurately discriminate early stage CC patients from HC subjects. These data demonstrated that serum PVT1 could be used as a novel noninvasive biomarker for the early diagnosis of CC. The combination of PVT1 with other lncRNAs, microRNAs and proteins would be more effective for the diagnosis of CC, and this hypothesis needs further investigation.

In addition to CC, we also measured serum PVT1 level in breast cancer and ovarian cancer patients. Serum PVT1 level is not changed in breast cancer and ovarian cancer patients, implying that the diagnostic value of PVT1 may be specific to CC. But serum PVT1 level in more types of cancers needs to be investigated.

In addition, we investigated serum PVT1 levels during the dynamics of CC. Our results revealed that serum PVT1 level was significant positive correlated with CC tissue PVT1 level. Serum PVT1 level was significantly reduced after surgery section of CC. These findings implied that serum PVT1 mainly derived from CC tissues and could be used for the monitor of CC dynamics.

Conclusions

We have observed that serum PVT1 level is mostly increased in CC patients, correlated with tumor size, FIGO stage, and lymph node metastasis. Serum PVT1 level shows high accuracy in discriminating CC patients from CIN patients and HC subjects, and also discriminating CIN patients from HC subjects. Serum PVT1 level indicates CC dynamics. Our findings demonstrate that PVT1 may be a novel noninvasive biomarker for CC early diagnosis.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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