Original Article

Potential New Urinary Biomarkers for Cervical Cancer Screening Using SELDI-TOF Mass Spectrometry

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Background: Urine samples offer certain advantages for clinical proteomic analysis, including their ease of collection using non-invasive procedures. Currently, there are several screening tests for cervical cancer, including conventional Pap smear and liquid-based cytology. Unfortunately, these techniques require cervical swabs to be performed that are more invasive and complex than urine collection.

Objective: The present exploratory study aimed to identify candidate urinary protein profiles that may be used as biomarkers to distinguish cervical cancer from human papillomavirus [HPV]-negative non-cervical cancer cases, using surface-enhanced laser absorption/ionization [SELDI] time-of-flight mass spectrometry [TOF MS].

Materials and Methods: Sixty urine samples from cervical cancer patients and HPV-negative women were subjected to analysis using SELDI-TOF MS and its associated software.

Results: The spectra of protein profiles used for analysis included mass-to-charge (m/z) values of 15,859, 33,385, and 66,730 Da. The peak at m/z 33,385 Da could distinguish between cervical cancer and HPV-negative non-cervical cancer cases with a sensitivity and specificity of 86.67% and 73.33%, respectively.

Conclusion: These results suggest that analysis of urine protein profiles by SELDI-TOF MS could potentially discriminate between cervical cancer and non-cervical cancer cases, and may thus be useful for developing novel screening tests for the detection of cervical cancer.

Keywords: Protein profiles, Cervical cancer, Urine proteomics, SELDI-TOF, HPV

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Cervical cancer is the second most common type of cancer in women in the world, after breast cancer, with an estimated 500,000 new cervical cancer cases every year[1]. It is a slow progressive disease that may take from one to several years to progress from a precancerous state to cancer. The precancerous state of cervical cancer is referred to as cervical intraepithelial neoplasia [CIN] and is categorized according to the levels of cell abnormality (CIN I, II and III)[2]. Approximately 80% of cervical cancers are found in developing countries, including Thailand[3]. Human papillomavirus [HPV] infection is a well-recognized risk factor for cervical cancer development. HPV can be classified into various types, with the high-risk HPV types such as HPV 16, 18, 52 commonly found in cervical cancer[4,5]. Currently, there are several screening tests for cervical cancer, including conventional Pap smear, liquid-based cytology and HPV DNA testing. Studies have suggested that the combination of liquid-based cytology and DNA testing has a high sensitivity for cervical cancer screening[6]. However, these techniques require cervical swabs to be performed that are more complex and invasive when compared with urine collection. Urine has thus become one of the most attractive specimens used for clinical proteomics, because of its ease of collection using noninvasive procedures[7].

Abnormalities in proteins and their interactions have been implicated in the etiologies of various diseases, including cancers. This has led to advances in proteomic technologies, which allow disease progression to be explored at the molecular level. Several studies have demonstrated the use of proteomic approaches to identify potential biomarkers for cancers[8-12]. Several proteomic methods have been used to identify disease biomarkers, including 2-dimensional [2D] gel electrophoresis, immunoblotting and/or mass spectrometry; however, most of these require multiple processing steps and time-consuming data analysis. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry [SELDI-TOFMS] is an affinity-based mass spectrometric analysis using specific chromatographic surfaces[13], which provides a potential clinical tool, especially in proteomics. It can be used to screen different patterns of protein expression in body fluids and tissue specimens, and the comparative analyses of data between patients and healthy subjects via rapid processing could provide for the identification of novel biomarkers. Previous studies have also demonstrated the benefit of this technology to provide protein biomarkers for classifying different types of precancerous states[14] and cervical cancer[15]. In the present study, SELDI-TOF MS was used to examine urinary proteomic biomarkers to detect multiple protein changes with simultaneously high sensitivity and specificity.

Materials and Methods

Sample preparation

This study was approved by the Ethical Committee for Human Research of Chulabhorn Research Institute (EC No. 31/2554). Urine samples were collected from 30 females with cervical cancer who were diagnosed at the Chulabhorn Hospital from July 2014 to April 2015, and 30 HPV-negative females. Protease inhibitor cocktails (Sigma-Aldrich, USA) were added to the 10 mL of freshly voided morning urine samples and fractionated within 24 hours. The urine samples were immediately centrifuged at 1,120 x g at 4°C for 5 minutes and the supernatants were subjected to acetone precipitation, adapted from the procedure described in Thongboonkerd V et al[16]. Briefly, 5 mL acetone was added to 5 mL supernatants, mixed by inverting, and stored in a refrigerator for more than 16 hours (overnight). The samples were then centrifuged at 1,120 x g at 4°C for 5 minutes and the supernatants were removed. The protein pellets were resuspended in denaturing buffer (8M urea, 1% CHAPS in PBS pH 7.2), aliquoted into 0.2 mL microcentrifuge tubes (fractionated urine samples) and stored at -80°C until analysis.

HPV genotyping

HPV genotyping was performed using the LINEARARRAY® HPV Genotyping Test (Roche, USA) to identify the HPV genotype in cytobrush cervical swabs. After DNA extraction, samples were subjected to several analytical steps, following the manufacturer's instructions. This technique was capable of identifying 37 HPV types, including 12 high-risk [HR], 8 probable high-risk [PR], and 17 low-risk [LR] types.

SELDI-TOF analysis

Fractionated urine samples were prepared in triplicate and diluted 1:10 in binding buffer (100 mM ammonium acetate pH 4.5, 0.1% Triton X-100) and applied to several types of protein chips (NP20, IMAC30, CM10, H50), according to the manufacturer's instructions (Bio-Rad Laboratories Inc., USA). The weak cation exchange protein chip [CM10] was selected for use on all samples as it provided more peaks and

higher intensities than the other chips.Samples were applied to the array chips,incubated in a humidity chamber for 1 hour, followed by shaking at 0.1 x g at room temperature. The array chips were then washed twice with binding buffer. Each spot was allowed to air dry and 0.5 µl of sinapinic acid (energy absorbing molecule) was added twice to each spot. CM10 arrays were analyzed using the SELDI-TOFMS analyzer (Bio-Rad, USA) following the manufacturer's instructions. Each urine samplewas examined in triplicate to generate protein profiles and data were compared using SELDI-TOF software.

Statistical analysis

Parameters between the cervical cancer group and HPV-negative group were compared using Student t-test and *p*-values <0.05 were considered to be statistically significant. The Wilcoxon's signed rank test was used to calculate *p*-values for SELDI-TOF data, to determine the significance of differences in peak intensities.

Results

Clinical data of cervical cancer patients and HPV-negative controls

Clinical data, including HPV genotypes and cancer stages are shown in Table 1. The HPV-negative controls were slightly younger than the cervical cancer group. Most of the cervical cancer patients (76.67%) had detectable high-risk HPV infection, and most had stage IIB disease (36.67%).

SELDI-TOF data of cervical cancer patients and HPV-negative controls

Among the eight identified spectra of protein profiles analyzed, three peaks (m/z of 15,859, 33,385 and 66,730 Da) were considered to have the potential to be useful as biomarkers. Details of the three analyzed protein spectra are shown in Table 2 and the relative expression levels of each peak are shown in Figure 1. The peaks at 16, 33 and 67kDa displayed higher intensities in the cervical cancer group than those of the HPV-negative controls (8.34, 11.59, and 55.89 vs. 0.93, 3.01, and 11.85, respectively). According to the peak intensities, we classified the protein spectra of the samples as positive or negative for each biomarker peak; samples showing a higher intensity than the cut-off value for each biomarker peak were classified as positive. Using this approach, we were able to distinguish between cervical cancer and HPV-negative cases with sensitivity, specificity, positive predictive value [PPV], and negative predictive value [NPV], as

Table 1. Clinical data of HPV-negative and cervical cancer patients

Parameters	HPV-negative	Cervical cancer
No. of patients	30	30
Median age (years)	49.5	54.5
Age range (years)	32 to 64	33 to 64
HPV genotyping	Negative	Type 16 (7, 23.33)
(No., % of cervical CA group)		Type 18 (5, 16.67)
		Type 31 (1, 3.33)
		Type 33 (2, 6.67)
		Type 52 (2, 6.67)
		Type 53 (1, 3.33)
		Type 59 (1, 3.33)
		Type 68 (2, 6.67)
		Type 72 (1, 3.33)
		>2 types of high risk-HPV (2, 6.67)
		Negative (6, 20.00)
CA state		IB (5, 16.67)
(No., % of cervical CA group)	-	IIA (3, 10.00)
		IIB (11, 36.67)
		IIIB (4, 13.33)
		IVA (1, 3.33)
		IVB (2, 6.67)
		Other with metastasis (4, 13.33)

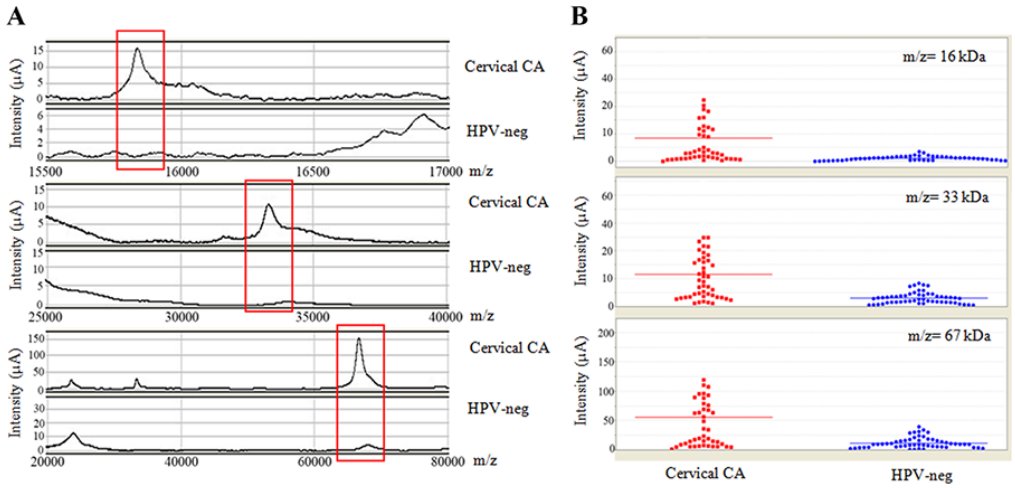

Figure 1. Peak intensities at m/z 16, 33 and 67 kDa of protein profiles obtained by SELDI-TOF MS, detected in the urine of cervical cancer patients and HPV-negative women.

Table 2. Details of analyzed protein peaks for classifying sample groups

Protein peak (Da)	*p*-value	Average intensity of protein peak (median ± SD)		Ratio
		HPV-negative	Cervical cancer	
15,859	0.00384	0.93±0.58	8.34±3.75	8.9
33,385	0.00004	3.01±1.06	11.59±4.96	3.9
66,730	0.00555	11.85±4.32	55.89±13.41	4.7

Table 3. Efficiency of analyzed protein peaks for distinguishing between control and cervical cancer

Parameter	m/z = 15,859 Da	m/z = 33,385 Da	m/z = 66,730 Da
Cut-off (intensity)	4.59	6.63	42.48
Sensitivity	76.67	86.67	73.33
Specificity	60.00	73.33	83.33
Positive predictive value [PPV]	65.71	76.47	81.48
Negative predictive value [NPV]	72.00	84.62	76.76

shown in Table 3. The 33 kDa peak showed the highest sensitivity and NPV, whereas the 66 kDa peak had the highest specificity and PPV among the three biomarker peaks.

Discussion

Thailand has a high incidence of cervical cancer, with an age-standardized rate of 17.8 per 100,000 person-years[3]. Screening of women at high risk of cervical cancer may thus help to decrease the incidence of this disease. However, patients may find commonly used screening methods, such as Pap smears using cervical swabs, uncomfortable and invasive, indicating the need for new screening biomarkers for cervical cancer. Such biomarkers may be identified through urinary proteomic analysis. Urinary biomarkers are currently used to detect several diseases, such as kidney disease, prostate cancer, and pancreatic cancer[17].

SELDI-TOF MS is a highly sensitive procedure that has previously been used to distinguish between

different types of several diseases, including hepatoblastoma, hepatocellular carcinoma, bladder cancer, ovarian cancer, and cervical cancer[17]. This technique can be applied to various different types of samples, including serum, plasma, saliva, protein lysates, and urine[17,18]. Piyathilake et al reported that SELDI protein profiles from plasma could be used to distinguish between controls and precancerous cases [CIN3] with 100% sensitivity and specificity[14]. In addition, a study from Taiwan reported that six plasma protein peaks from SELDI-TOF analysis could be used to discriminate between early invasive cervical cancers (carcinoma in situ and squamous cell carcinoma) and controls, with 91% sensitivity and 97% specificity[19]. Moreover, Van Gorp et al reported that analysis of serum protein profiles could distinguish controls from different types of cervical cancer, including lymph node metastasis, lympovascular involvement, and recurrence, with high sensitivities and specificities[20]. A Chinese study of biomarkers of neural tube defects from different types of samples, including serum, amniotic fluid, and urine, showed that protein profiles from all the different types of samples could differentiate between neural tube defect and control cases with high sensitivity and specificity (>80%)[21]. Interestingly, Mu et al used lectin-captured glycopeptide chips to analyze urine samples by SELDI-TOF MS, and distinguished ovarian cancers from cervical and endometrial cancers with high sensitivity and specificity (100%)[15]. These data support the use of SELDI-TOF MS for screening protein profiles and identifying potential biomarkers for cancers.

To the best of our knowledge, this is the first study to use SELDI-TOF MS to analyze and compare urinary proteomic profiles from patients with cervical cancer and non-cervical cancer. Our results suggest that SELDI-TOF analysis of urinary protein profiles could be used to discriminate between these cases. Although the sensitivity and specificity were less than 90%, this method can further be developed using different types of protein chips, as demonstrated by the use of protein chips by Mu et al to study glycoproteins[15].

Conclusion

We identified the protein peak at m/z 33,385 Da by SELDI-TOF MS that could distinguish between cervical cancer and HPV-negative non-cervical cancer cases with a good sensitivity and specificity, suggesting that SELDI-TOF analysis of urinary protein profiles may represent a potential tool for the development of a non-invasive screening test for the detection of cervical cancer.

What is already known about this topic?

SELDI-TOF protein profiles have been used as a screening method to classify several types of pre-malignancies or types of cervical cancers. In addition, some studies have applied the technique to serum or plasma proteins to classify precancerous or cervical cancers. One previous study applied SELDI-TOF protein profiles to distinguish ovarian cancer from cervical cancer and endometrial cancer.

What this study adds?

This is the first study to demonstrate the use of SELDI-TOF MS to analyze urinary proteomic profiles in patients with cervical cancer and non-cervical cancers.

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Potential conflicts of interest

The authors declare no conflicts of interest.

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