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Urine surface-enhanced Raman spectroscopy combined with SVM algorithm for rapid diagnosis of liver cirrhosis and hepatocellular carcinoma

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ABSTRACT

In this paper, we investigated the feasibility of using urine for surface-enhanced Raman spectroscopy (SERS) for the rapid screening of patients with liver cirrhosis and hepatocellular carcinoma (HCC). The SERS spectra were recorded from the urine of 49 liver cirrhosis, 55 HCC, and 50 healthy volunteers using a Raman spectrometer. The normalized mean Raman spectra showed the difference of specific biomolecules associated with the illnesses, and the metabolism of specific nucleic acids and amino acids is abnormal in patients with liver cirrhosis and HCC. Based on the SVM algorithm, the urine SERS method could identify liver cirrhosis (sensitivity 88.9%, specificity 83.3%, and accuracy 85.9%) and HCC (sensitivity 85.5%, specificity 84.0%, and accuracy 84.8%). It has a higher diagnostic sensitivity for HCC than serum Alpha fetoprotein (AFP). This exploratory study showed that the urine SERS spectra combined with the SVM algorithm has indicated great potential in the noninvasive identification of liver cirrhosis and HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third leading cause of cancer death in the world by 2020, with approximately 906,000 new cases and 830,000 deaths [1,2]. Clinical studies have confirmed that liver cirrhosis is linked to the development of HCC, and that most HCCs are caused by liver cirrhosis [3]. Early HCC, on the other hand, has a slow development, and symptoms such as portal hypertension, liver damage, and even multiple systems are involvement do not occur until the situation has severely progressed, most patients are diagnosed in the middle and late stages of their illness, missing out on the best treatment options [4]. Thus, it's critical to increase the accuracy of early HCC detection and better the differential diagnosis of liver cirrhosis and HCC.

For clinical diagnosis of liver illnesses, B-scan ultrasonography, MRI (Magnetic Resonance Imaging), CT (Computed Tomography) and serological examination methods are being used. The imaging methods can assess lesions through a variety of reconstruction modes, but they all have a number of drawbacks, including difficulties standardizing and early detection of lesions. The technical competence and experience of doctors are intimately tied to these procedures. The inspection procedures are complicated, time-consuming, costly and they expose the patient to radiation [5]. Typically employed indications in serological testing, such as Alpha fetoprotein (AFP), have drawbacks, such as low diagnostic sensitivity [6,7]. As a result, finding a speedy and highly accurate HCC detection method is critical in clinical practice.

Raman spectroscopy is a label-free, rapid, and non-invasive optical detection technique [8,9]. However, in the actual application process, Raman spectroscopy has the disadvantages of low signal intensity and small scattering cross section, which limits its application in molecular detection. Surface Enhanced Raman Scattering (SERS) can compensate for standard Raman scattering's limited sensitivity [10]. When the

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biomolecules are adsorbed onto the gold or silver nanoparticles surface, yielding rich chemical fingerprint information of biomolecules, such as DNA, RNA, protein and lipid [11]. SERS has recently attracted increased interest in biomedical research, and it is expected to replace the existing conventional diagnostic technology and emerge as a new medical diagnostic method [12]. SERS is emerging as a potentially useful clinical adjunct in cancer detection, capable of identifying the subtle changes associated with cancer progression in biological samples (cell, blood, and urine etc.) [13]. It should be highlighted that human urine is a more ideal medium for cancer detection than blood because it is noninvasive, plentiful, and easy to collect. Importantly, urine is the product of human metabolism from the blood, which makes it contains many biological molecules (e.g., protein, DNA, and RNA etc.) [14] that can timely reflect human's health condition. Recent studies have reported the significance of the application of urine SERS in cancer detection for prostate cancer [15], breast cancer [14] and esophageal cancer [16]. These exploratory work shows that the urine SERS method is very promising for cancer detection. However, urine SERS has not been used for the diagnosis of liver cirrhosis and HCC.

This study evaluated the feasibility of applying SERS spectra based on silver nanoparticles for label-free detection of urine samples from patients with liver cirrhosis, HCC, and healthy volunteers. The SVM algorithm was used to establish the diagnosis models. This preliminary study may develop a fast, convenient and non-invasive method for the detection of liver cirrhosis and HCC.

2. . Materials and methods

2.1. Collection of urine samples

The First Affiliated Hospital of Xinjiang Medical University provided a total of 154 urine and serum samples for this study. There were 49 cases of liver cirrhosis, 55 cases of HCC, and 50 healthy volunteers, according to the clinical diagnosis. Table 1 contains basic demographic information about the participants, such as their age and gender etc. The level of serum AFP was detected by Abbott's automatic chemiluminescence immune analyzer (Abbott i2000SR) and supporting Kit (Abbott 3P36/07P90). The detection steps were carried out in strict accordance with the instructions of the kit. The cut-off value of AFP for HCC was set at 20 ng/ml, according to a previous study [17]. The present study protocol was ethically approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (Ethical Approval Number: No. K202108–04). Urine samples were collected in accordance with the routine procedures of clinical laboratories, centrifuged at a speed of 4000 rpm for 5 min before the experiment, and stored in a refrigerator at-80 °C.

2.2. . SERS measurements

The urine samples were thawed at room temperature before the SERS measurement, and then 5 μl urine sample was mixed with 5 μl Ag colloid in 1:1 proportion, and dropped onto aluminum foil paper for spectral acquisition. Ag colloid was purchased from Nanjing Jianzhi Instrument Equipment Co., Ltd. A Raman micro-spectrometer (ATR8300MP, Optosky, China) equipped with a 785 nm diode laser was used for the measurement of urine SERS spectra in the range of 400–1800 cm^{-1}. A 20x microscope objective lens (NA = 0.40) was used, and the laser power and integration time were 30 mW and 5 s, respectively. A total of 154 serum SERS spectra were obtained, including 49 spectra of liver cirrhosis, 55 spectra of HCCs, and 50 spectra of healthy volunteers.

Table 1
The simple information of patients with liver cirrhosis, HCC, and healthy volunteers.

	Liver Cirrhosis (n = 49)	HCC (n = 55)	Healthy volunteers (n = 50)
Age			
Mean	56.1	54.3	46.2
Median	54.5	52.5	47
Gender			
Male	27	34	23
Female	22	21	27
AFP (ng/ml)			
≤ 20	46	36	50
> 20	3	19	0

2.3. . SERS data processing and SVM classification

The preprocessing of urine SERS spectral data includes smoothing and denoising, baseline correction and normalization operations. This project used the Savitzky-Golay (SG) algorithm to smooth the collected urine spectrum data to reduce the noise, and the baseline was subsequently removed by the adaptive iteratively reweighted penalized least squares (airPLS) algorithm [18]. Performed vector normalization processing on each spectrum, and then used the SVM algorithm to build the diagnostic models. The C-SVM model based on LIBSVM package (version 3.22) was adopted for SVM classifications [13]. Two commonly used kernel functions, namely linear, and Gaussian radial basis function (RBF) were used to build classification models. All programs were implemented in MATLAB language (version MATLAB R2020a). Each model was evaluated by the leave-one-out cross-validation (LOOCV) method. The diagnostic accuracy, specificity and sensitivity of each model are analyzed and compared to the established different classification diagnostic models.

3. . Results and discussion

3.1. Spectral preprocessing results

The spectral preprocessing of a representative urine sample from a healthy population was shown in Fig. 1. To begin, denoising processing is required to improve the spectral signal in the spectral collecting procedure of a urine sample due to instrument noise and external ambient noise. Second, because the Raman signal is accompanied by the formation of the autofluorescence signal in biological material, the spectrum must be corrected for baseline. Finally, vector normalization was applied to each spectrum.

3.2. The mean SERS spectra of the three types of urine samples

The normalized mean SERS spectra of urine from cirrhosis patients, HCC patients, and healthy volunteers were shown in Fig. 2(a). The average SERS spectra of the three groups of urine had main characteristic peaks at 374, 495, 556, 641, 716, 889, 1002, 1090, 1130, 1220, 1282, 1358, 1455, 1596 and 1706 cm^{-1}. To highlight the spectral differences among the three groups, the difference spectra were shown in Fig. 2(b). SERS spectra of urine are dominated by different vibration modes of biomacromolecule (proteins, glucose, DNA/RNA, etc.) [15, 19]. Thus, we tentatively assigned the SERS spectral data obtained by the experiment, as shown in Table 2, to better analyze SERS characteristics of biomolecules in urine.

As can be seen from Fig. 2(b), The biomolecules content corresponding SERS bands at 495, 556, 716, 889, 1130, 1220, 1282, 1455, 1596 and 1706 cm^{-1} were up-or down-regulated in the liver cirrhosis group and HCC groups compared to the healthy group. The spectrum of differences between groups, with larger differences between HCC and normal groups, liver cirrhosis group and normal groups than between HCC and liver cirrhosis. At the SERS peaks of Adenine (556 cm^{-1}), Guanine (716 cm^{-1}), Deoxyribose (889 cm^{-1}), Uric acid (1130 cm^{-1}) and Uracil (1455 cm^{-1}) the characteristic peaks of liver cirrhosis and HCC group were lower than those of healthy group, suggesting that there is abnormal metabolism of cancer cell with the breakage of DNA/

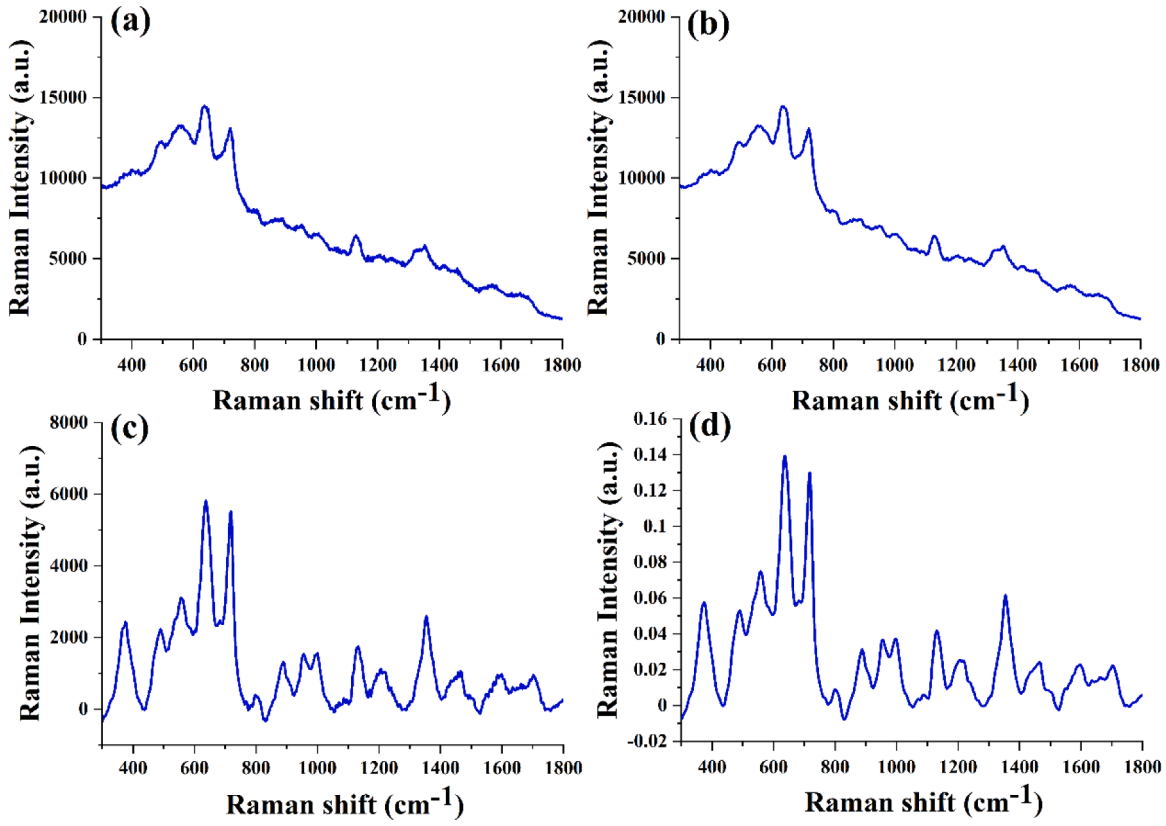


Fig. 1. SERS spectral preprocessing results of a selected representative sample;(a) Raw spectrum; (b) The spectrum of the raw spectrum after denoising;(c) Baseline corrected spectrum;(d) Normalized spectrum.

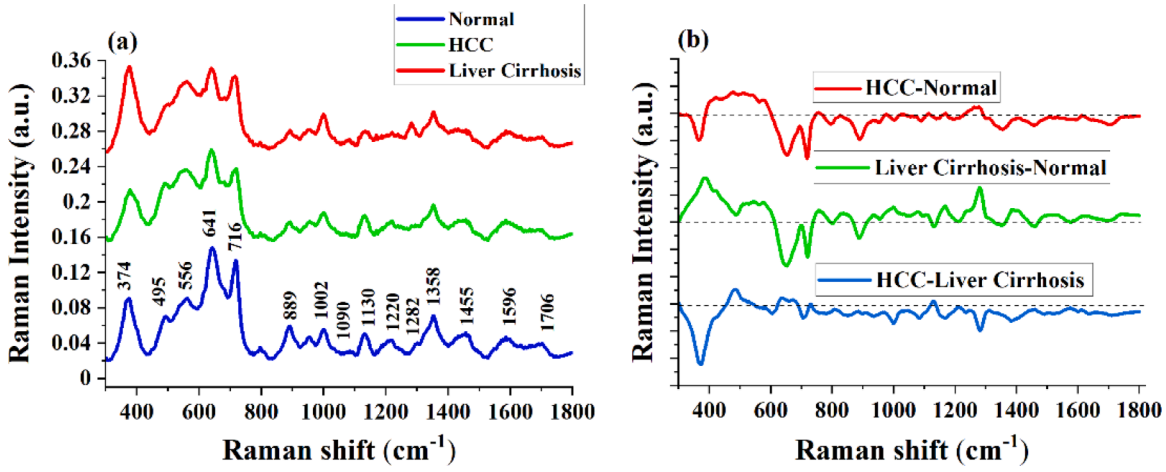


Fig. 2. (a) Comparison of the normalized average SERS spectra from liver cirrhosis, HCC and healthy volunteers' urine samples; (b) The difference spectra calculated from the mean SERS spectra among the three groups.

RNA strands bases in the urine of cancer patients, which directly affects the purine metabolism of the body and changes the content of nucleic acid and uric acid. Similar changes were observed in urine samples of gastric cancer and breast cancer [14,21,22]. Nucleic acid metabolites in urine can be used as novel tumor markers, which have been reported in many studies [23,24]. The increase of modified nucleosides in the urine of HCC patients was related to the rapid growth, tissue destruction and RNA metabolism of liver tumors [25]. In addition, these changes were also mentioned in the urine metabolism studies of patients with HCC and liver cirrhosis [16,26].

Apart from that, the SERS bands at proline (374 cm^{-1}), Urea (1002 cm^{-1}), histidine (1090 cm^{-1}), Serine (1220 cm^{-1}), Tryptophan (1358 cm^{-1}), alanine (1596 cm^{-1}), and Creatinine (1706 cm^{-1}) were up-or down-regulated in the liver cirrhosis group and HCC group, implying abnormal nucleic acids and amino acids metabolism. The liver was the body's primary location of amino acid metabolism. Damage to the liver from a variety of causes can result in complex metabolic abnormalities in the body [27]. The liver's intake, metabolic inactivation and excretion of amino acid metabolites will inevitably occur obstacles to the metabolism of amino acids in liver cells, which in turn leads to increased blood amino acid concentration, and loss from urine, which affects the changes in the level of amino acids in blood, urine and bile [28].

Table 2
The tentative assignments for urine SERS spectra [2,15,20,29–37].

Raman shift (cm^{-1})	Tentative assignment
374	Proline
495	Glycogen, Guanine, Tryptophan
556	Adenine
641	Citric acid
716	Guanine
889	Deoxyribose, Glycine
1002	Urea
1090	Phosphate, Histidine
1130	Uric acid
1220	Serine
1282	Glutamate
1358	Guanine, Tryptophan
1455	Uracil
1596	Alanine, Serine
1706	Creatinine

Therefore, analyzing SERS peak intensities can reveal specific and subtle changes in biomolecules in cancer urine samples, allowing us to look for new biomarkers and thereby improve cancer detection. The alterations in the urine SERS spectra of patients with liver cirrhosis, HCC and healthy volunteers indicated above suggest that the urine-based SERS detection method may become a novel technology for rapid and simple detection and diagnosis of liver diseases.

3.3. Classification results based on SVM

After preprocessing each spectrum, the SVM algorithm was used to create the classification models, and the LOOCV method was used to verify the performance of each model. The detailed results of the SVM based on LOOCV method to classify the three groups were shown in Table 3. For the 50 healthy samples, 1 case was incorrectly classified as liver cirrhosis, 8 cases were incorrectly classified as HCC. Of the 49 cases of liver cirrhosis, 7 cases were incorrectly classified divided as healthy, 9 cases were incorrectly classified as HCC. Of the 55 cases of HCC samples, 11 cases were classified as cirrhosis and 7 cases were classified as healthy. Therefore, the diagnostic sensitivity of the three groups was 82%, 67.3%, and 67.3%, specificity of 86.4%, 84.6%, and 83.8%, respectively [38]. The overall accuracy rate of the SVM was 72.1%.

We looked into the effect of the urine SERS combined with the SVM algorithm on binary classification. Table 4.showed the binary classification results of the healthy group and the liver cirrhosis (sensitivity 88.9%, specificity 83.3%, and accuracy 85.9%), healthy group and HCC group (sensitivity 85.5%, specificity 84.0%, and accuracy 84.8%), liver cirrhosis group and the HCC group (sensitivity 79.6%, specificity 76.0%, and accuracy 77.9%), healthy group and the case group (Liver cirrhosis and HCC group, sensitivity 92.0%, specificity 77.8%, and accuracy 87.0%). The reason for the poor classification between liver cirrhosis and hepatocellular carcinoma patients might be the components in the urine of liver cirrhosis and hepatocellular carcinoma patients were similar. About 80% of the hepatocellular carcinoma was derived from liver cirrhosis, and both diseases have similar serology, and urine metabolic indexes [39]. Zhao et al [40] found that incorporating patient demographics into the Raman spectroscopy algorithm could improve *in vivo* skin cancer diagnostic specificity. We used Zhao's method and found that the accuracy of the classification between liver cirrhosis and hepatocellular carcinoma patients (80.8%) was improved, which was about 3% higher than the original. The serum level of AFP was commonly applied into the clinical diagnostic of HCC. Its sensitivity for HCC was weak (22%−60%), and its specificity was perfect (93%−97%) [41–43]. It has been reported that using serum AFP levels in conjunction with abdominal ultrasound can significantly improve the early detection of HCC [44]. Thus, we want to improve the diagnostic model's classification accuracy by increase the sample size of each group, combining Raman spectroscopy with AFP, imaging data, and more clinical demographic variables in the next step.

Table 3
Cross validation results of SVM based on Gaussian radial basis function.

	Actual group	LOOCV		
		Healthy	Liver Cirrhosis	HCC
TRUE	Healthy	41	1	8
	Liver Cirrhosis	7	33	9
	HCC	7	11	37
	Sensitivity	82%	67.3%	67.3%
	Specificity	86.4%	84.6%	83.8%

Table 4
Sensitivity, specificity, and accuracy for the difference comparison groups after binary classification.

Comparison group	Sensitivity (%)	Specificity (%)	Accuracy (%)
Healthy *vs* Liver cirrhosis	88.9	83.3	85.9
Healthy *vs* HCC	85.5	84.0	84.8
Liver cirrhosis *vs* HCC	79.6	76.0	77.9
Healthy *vs* Case group [a]	92.0	77.8	87.0

[a] Case group, Liver cirrhosis and HCC group.

In this study, the sensitivity of the serum AFP for HCC was 34.5%, which was lower than the urine SERS combined with the SVM algorithm. The results indicated that combination of urine SERS and SVM algorithm had great potential in accurately identifying healthy people, patients with liver cirrhosis and HCC, and has high sensitivity and specificity.

4. Conclusion

In summary, this study was based on the potential unlabeled urine detection of SERS and combined with machine learning to achieve rapid diagnosis of liver cirrhosis and HCC. The findings of this study revealed that Raman spectroscopy data of healthy volunteers, liver cirrhosis patients, and HCC patients may be collected with only a minimal amount of urine using a Raman spectrometer, and there were significant spectral differences among healthy, liver cirrhosis, and HCC. The SVM algorithms were used to classify SERS spectra of the different urine types with high diagnostic sensitivities and accuracy, demonstrating the great potential of urine SERS for rapid, convenient and noninvasive detection of liver cirrhosis and HCC. To further evaluate the viability of this technology, we will gather more SERS data from more specimens, including HCC and liver cirrhosis urine.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest related to this article.

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