Raman spectroscopic identification of normal and malignant hepatocytes

Jianyu Guo (郭建宇), Bing Du (杜 冰), Min Qian (钱 旻), Weiying Cai (蔡炜颖), Zugeng Wang (王祖赓), and Zhenrong Sun (孙真荣)*

State Key Laboratory of Precision Spectroscopy (East China Normal University), Department of Physics, and Department of Biology, East China Normal University, Shanghai 200062

**E-mail: zrsun@phy.ecnu.edu.cn*

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Raman spectroscopy has strong potential for providing non-invasion diagnosis of cancers. In this paper, micro-Raman spectroscopy is used to diagnose one most common liver cancer, hepatocellular carcinoma (HCC). The statistical analyzes, including *t*-test, principal component analysis (PCA), and linear discriminant analysis (LDA), are performed on the Raman spectra of malignant and normal hepatocytes. The *t*-test-LDA results show that the 786- and 1004-cm^{-1} bands of the malignant and normal hepatocytes are significantly different, and PCA-LDA results show an overall accuracy of 100% for the Raman spectroscopic identification of normal and malignant hepatocytes in our experiment.

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On a global scale, liver cancer is one of the leading causes of cancer morbidity and mortality. Hepatocellular carcinoma (HCC) is the predominant histologic subtype of liver cancer with an estimated annual incidence of one million cases worldwide, particularly in Asian countries[1−3]. It is one of the most lethal malignancies, and is also one of the most prevalent malignant diseases of adults in China[3,4]. So, it is important to realize the early diagnosis of HCC.

Many methods have been developed to detect the neoplastic changes. Optical spectroscopies, such as fluorescence spectroscopy, infrared (IR) spectroscopy, and Raman spectroscopy, have been the active fields in recent years. However, fluorescence spectroscopic technique lacks specific structural information because the fluorescence spectra of biological species are similar due to the presence of some common fluorophores[5]. Although IR spectroscopy can provide detailed structural information, it is not suited for an aqueous environment due to the strong absorption of water. Raman spectroscopy has several advantages over fluorescence and IR spectroscopies. It is a non-invasive method to investigate the biological samples and can attain the essential information about healthy and pathological tissues. Raman spectroscopy has recently shown promise in the diagnosis of bladder, prostate, stomach, larynx, tonsil, lung, and esophagus cancers, as well as basal cell carcinoma[6−13]. Despite of these advantages, very few studies on the Raman spectrum of HCC have been reported[14]. In this paper, the aims are focused on exploring whether Raman spectroscopy, associated with statistical analysis method, is able to identify normal and malignant hepatocytes with a high accuracy, and obtain more about the spectral differences between the normal (HL-7702) and malignant (BEL-7402) hepatocytes.

The hepatocellular carcinoma cell line BEL-7402 and the normal liver cell line HL-7702 were all purchased from the cell bank of Shanghai Institutes for Biological Sciences (Shanghai). The cell lines of BEL-7402 and HL-7702 were cultured in RPMI 1640 with 10% fetal calf serums (Invitrogen, Carlsbad, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. Then, the cells were digested with 0.25% trypsin, resuspended in serum-containing medium to 10^4 cells/ml, and grown in 24-well chamber slides overnight. Finally, the cells on quartz slides were picked out and washed three times with phosphate buffered saline (PBS, 0.01 mol/L, pH7.2) to remove all growing medium.

Raman spectra of BEL-7402 and HL-7702 were recorded by a confocal micro-Raman spectrometer (T64000, HORIBA Jobin Yvon, France), equipped with a 50-mW argon-krypton laser excitation source at 514 nm, a microscope (IX 81, Olympus, Japan), a holographic notch filter to reject Rayleigh scattering and a liquid nitrogen cooled charge-coupled device (CCD) detector (CCD-3000, HORIBA Jobin Yvon, France). The spectrometer was calibrated with the silicon phonon mode at 520 cm^{-1}. A 100× microscope objective (numerical aperture NA=0.95, Olympus, Japan), which produced a laser spot size of about 1 μm, was used to focus laser and collect Raman scattering on a single cell. The power at the sample was about 2 mW to ensure that no sample degradation occurred. Raman spectra in the range of $600 - 1800$ cm^{-1} were recorded with an integration time of 100 s. These experimental conditions were kept constant for all measurements. The Raman spectrum of a cell was calculated as the average of five measured Raman spectra, and 29 malignant cells and 21 normal cells were measured with Raman micro-spectroscopy.

Spectra acquisition and all preliminary spectral correction such as smoothing, baseline subtraction, normalization, and spectral analysis were carried out using the Labspec6.0 software. The spectra were preprocessed by five-point smoothing to reduce noise. The Raman spectrum baseline was corrected by fitting and subtracting a fifth-order polynomial function. Then, the baseline corrected Raman spectra were normalized in the range of $600 - 1800$ cm^{-1}.

Table 1. Assignments of the Raman Bands for HL-7702 and BEL-7402 Cells

Peak	Assignment[15,16,19–24]			
(cm^{-1})	Protein	DNA/RNA	Lipid	Carbohydrate
786		O–P–O Str.		
825	Tyr out-of-Plane Ring Br.	O–P–O Str.		
852	Tyr Ring Br.			
1004	Phe Sym. Ring Br.			
1032	Phe C-H in-Plane			
1082	C–N Str.	PO_2^- Str.	C–C Str.	C–O Str.
1126	C–N Str.		C–C Str.	C–C Str.
1173	Tyr C–H in-Plane Bend			
1207	Trp, Phe C–C_6H_5 Str.			
1230 – 1280	Amide III			
1311	CH_3CH_2 Wagging	G	CH_3CH_2 Wagging	
1339	C–H Def.	G, A		
1360			CH_3 Sym. Str.	
1585		G, A		
1610	C=C Phe, Tyr			
1656	Amide I			

Str.: stretching mode; Br.: breathing mode; Def.: deformation mode; Sym.: symmetric; A: adenine; G: guanine; C: cytosine; Phe: phenylalanine; Tyr: tyrosine; Trp: tryptophan

The statistical methods including t-test, principal component analysis (PCA), and linear discriminant analysis (LDA) were used to analyze the Raman spectra for the cells. By PCA, the large number of observed spectral vectors from various samples can be resolved into linear combinations of the few independent basis vectors called principal components or factors. In our analysis, PCA was carried out on the spectra in the range of $900-1700$ cm^{-1}. Ten principal components were used to explain 90% of total variance in the Raman spectra and were further analyzed for discriminating between two cell lines using LDA.

The mean Raman spectra of HL-7702 and BEL-7402 cells in the region of $600-1800$ cm^{-1} are shown in Fig. 1. The similar bands can be assigned to nucleic acids, proteins, lipids, and carbohydrates[15,16]. The strong bands at 1447 and 1656 cm^{-1} can be attributed to the CH_2 deformation mode and the C=C stretching mode of the lipids and proteins, respectively. The band originating at 786 cm^{-1} can be assigned to the O–P–O stretching mode of DNA. The bands appearing at 1004 and 1032 cm^{-1} can be assigned to the symmetric ring breathing mode and the C–H in-plane bending mode of phenylalanine, respectively. The assignments for the observed Raman bands are listed in Table 1. The prominent protein bands at 1656 and $1230-1280$ cm^{-1} can be used to determine the secondary structure and the content of protein[17,18].

As shown in Fig. 1, the spectra are characterized by the nine major bands at 786, 825, 852, 1004, $1230-1280$, 1311, 1447, 1585, and $1625-1720$ cm^{-1}. Here, to study where there are evident differences between the malignant and normal hepatocytes, the t-test is performed on the nine bands, yielding one P-value per frequency. The P-value less than 0.05 indicates that there is significant Raman spectroscopic difference between the malignant and normal hepatocytes, whereas the P-value above 0.05 indicates that there is no significant Raman spectroscopic difference. The results of t-tests for the bands show that the intensities of these bands are significantly different between two cell lines, except for the 1585 ($P = 0.15$) and $1625-1720$ cm^{-1} ($P = 0.40$) bands. As a further progress, the seven significantly different bands with the P-values less than 0.05 are used for LDA. The LDA results (Table 2) show that the correct classification for normal hepatocytes can be obtained to be 100%. For the malignant hepatocytes, the accuracy is 82.8%. The absolute value of the standardized coefficient from LDA, which indicates the relative contribution of each band[25], are 0.77, 0.069, 0.034, 0.41, 0.052, 0.11, and 0.11 for the bands at 786, 825, 852, 1004, $1230-1280$, 1311, and

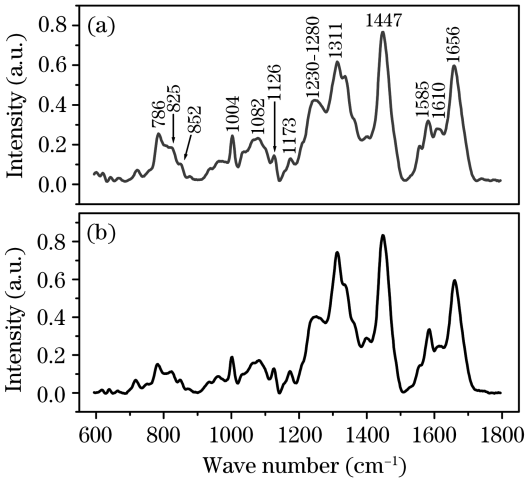

Fig. 1. Mean Raman spectra for (a) malignant (BEL-7402) and (b) normal (HL-7702) hepatocytes.

Table 2. Cross-Validation Classification Results

	Group	Predicted		Total
		Malignant	Normal	
Count	BEL-7402	24	5	29
	HL-7702	0	21	21
Accuracy (%)	BEL-7402	82.8	17.2	100.0
	HL-7702	0.0	100.0	100.0

1447 cm^{-1}, respectively. Thus, the largest absolute value of standardized coefficient is 0.77, which reveals that the band at 786 cm^{-1} is significantly different between the two cell lines and is the most crucial in distinguishing between the malignant and normal hepatocytes. The band intensity at 786 cm^{-1}, assigned to O–P–O, appears to be higher for malignant hepatocytes than that for normal hepatocytes. The larger intensity of O–P–O in malignant hepatocytes may indicate that there are more DNA molecules in malignant cells than in normal cells. According to the absolute value of standardized coefficient, the band at 1004 cm^{-1} is the second crucial parameter for the discrimination between the malignant and normal hepatocytes. The intensity at 1004 cm^{-1} is stronger for malignant hepatocytes than that for normal hepatocytes. It may reflect a vital change in the environment of the phenylalanine residue of hepatocyte proteins. The canceration of cells may disrupt the hydrogen bonds and the protein may unfold, and therefore the phenylalanine side chain may become more "exposed". So the phenylalanine in the malignant hepatocytes can generate a larger Raman scattering at 1004 cm^{-1} than that in normal hepatocytes. The t-test results show that the P-value of the amide I band ($1625 - 1720$ cm^{-1}) is more than 0.05, which suggests that the secondary structure of protein is not significantly different between the malignant and normal hepatocytes. However, the P-value of amide III ($1230 - 1280$ cm^{-1}), which is more sensitive to the structural changes than the amide I band, is less than 0.05. The results suggest that the secondary structure of protein is possible to be different between the malignant and normal hepatocytes.

Multivariate statistical analysis can highlight the delicate differences between the samples. PCA and LDA recently have been successfully used to analyze the Raman and IR spectra[26−32]. In order to discriminate between normal and malignant hepatocytes, all 50 Raman spectra were analyzed by PCA and LDA. In this paper, ten principal components from PCA are used to feed the LDA model by leave-one-out cross-validation, and then one discriminant function can be obtained. This process provides a powerful method to produce high predictive diagnostic accuracy as to distinguish between the malignant and normal hepatocytes well. As shown in Fig. 2, the LDA scores for BEL-7402 (29 samples) and HL-7702 (21 samples) cells are separated into two clusters, and an appreciable distinction has been made between the two groups of cells. As shown in Table 3, 100% of original grouped cells and 100% of cross-validated grouped cells are correctly classified. The results show the high accuracy (100%) for Raman spectroscopic identification of normal and malignant hepatocytes based on PCA and LDA.

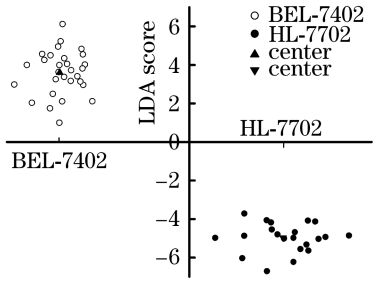

Fig. 2. LDA scores for normal (HL-7702) and malignant (BEL-7402) hepatocytes.

Table 3. LDA Classification Results from Principle Components

	Group	Predicted		Total
		Malignant	Normal	
Count[1]	BEL-7402	29	0	29
	HL-7702	0	21	21
Accuracy (%)[1]	BEL-7402	100.0	0.0	100.0
	HL-7702	0.0	100.0	100.0
Count[2]	BEL-7402	29	0	29
	HL-7702	0	21	21
Accuracy (%)[2]	BEL-7402	100.0	0.0	100.0
	HL-7702	0.0	100.0	100.0

[1]For the original grouped case; [2]for the cross-validation case.

In this paper, the experimental results indicate that micro-Raman spectroscopy is a useful tool for screening one most common liver cancer, HCC, and a powerful tool for investigating the biochemical modifications in cellular component and content. Statistical analyzes including t-test, PCA, and LDA can improve the Raman spectroscopic identification. The t-test-LDA approach, which is more suitable for understanding biological changes in cells, show that the bands assigned to O–P–O and phenylalanine are significantly different between the two cell lines and the most crucial in distinguishing between the malignant and normal hepatocytes. The accuracy of 100% can be achieved by PCA-LDA method to discriminate between malignant and normal hepatocytes in our experiment.

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