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Raman spectroscopic analysis of apoptosis of single human gastric cancer cells

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ABSTRACT

A drug (5-FU) was employed to treat the gastric carcinoma cells and induce apoptosis of the cancer cells. Raman spectra obtained from single gastric carcinoma cells and the induced apoptotic cells through scan-excitation mode were used to analyze the effectiveness of the treatment. The major difference of the apoptotic cells from the cancer cells are the reduction in intensities of vibration bands generated by cellular lipids, proteins and nucleic acids. In particular, large intensity reduction in nucleic vibrations at 782, 1092, 1320, 1340, and 1578 cm^{-1} was observed upon apoptosis of the gastric carcinoma cells. Up to 45% reduction in the magnitude of the 782 cm^{-1} peak in Raman spectra of the apoptotic cells was observed, which suggests the breakdown of phosphodiester bonds and DNA bases. We showed that the principal components analysis (PCA), a multivariate statistical tool, can be used to distinguish single apoptotic cells and gastric carcinoma cells based on their Raman spectra.

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1. Introduction

Stomach cancers are the fourth in the common cancers and the second that causes worldwide [1]. Countries with very high incidence of stomach cancers include China, southern India, Japan, Belarus and Costa Rica [1]. The incidence and mortality in the risk of stomach cancer has been declining worldwide [1,2]. The animal models would greatly facilitate the development of treatments or inoculations for many diseases [2]. However, there are many differences between animal models and human beings when they are treated with drug. Therefore, it is beneficial to choose the human gastric cancer cells to explore the mechanism of cancer apoptosis and to generate scientific knowledge for treatment of the diseases with respect to animal models.

Apoptosis, defined as a programmed cell death (PCD) [3], is a distinct, intrinsic cell death program that occurs in various physiological and pathological situations [4,5]. It was first described by Kerr et al. [24]. The basic process of apoptosis in vitro is based on the changes of chromatin and nucleolus distribution in the plasma membrane. The nuclear morphological changes could be classified into three stages during apoptosis. In stage I, nucleolus is rippled or creased, and a part of chromatin is condensed. In stage IIa, chromatin of the cell's nucleolus is highly compacted and fragmented. In stage IIb, nucleolus fracture completes and the cell becomes apoptotic body. Apoptosis is controlled by cellular gene. The regulation gene can be classified into two classes: one inhibits apoptosis and the other induces apoptosis. Most importantly, the regulation gene could spurs or induces apoptosis of cancer cells; this could provide a new approach to treat the cancer. With the understanding of the mechanism of cancer apoptosis, a variety of drugs will be used to induce cancer apoptosis and to accelerate the death of cancer cells, which could become a promising method for cancer treatment.

The test of apoptosis is usually based on the examination of morphology and biochemical characteristics of the apoptotic cells, especially the rupture of DNA .The apoptosis analysis techniques include the observation of cell morphology, DNA electrophoresis, comet electrophoresis, TUNEL, and flow cytometry. But these methods usually need the addition of staining dye or fluorescent labeling, which may damage the cells and cause unwanted effect, when testing the effect of the treatment drugs. In addition, flow cytometry method needs many samples for analysis and filtration on the level of single cells, which requires time-consuming sample preparation in the clinical application [6].

Raman spectroscopy is based on inelastic scattering of photons by vibrating molecules or crystal lattice. It was India physicist C.V. Raman who in 1928 first found the famous phenomenon of the Raman effects [7]. Raman spectroscopy is a non-destructive

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technique that provides information about the molecular structure of the sample to be investigated. The positions, intensities, and line-widths of the various spectral bands can be used to probe primary, secondary, tertiary, and quaternary structures of large biological molecules [8]. Raman spectroscopy has become a powerful analytical technique in different areas of physics, chemistry, biology and interdisciplinary fields [10,11]. Raman spectroscopy, especially micro-Raman spectroscopy has been successfully applied for the analysis of a variety of single cells, including cancer cells. In addition, chemical-contrast imaging of cancer cells has been obtained by Raman mapping or coherent anti-Stokes Raman scattering (CARS) imaging, which is based on the spatial distribution of a specific vibrational band in the cell. To the best of our knowledge, there is no report on the identification of apoptosis of gastric carcinoma cancer cells following the treatment with 5-Fu drug using single cell-Raman spectroscopy.

In this study, we applied near-infrared (NIR) Raman spectroscopy fot he analysis of apoptosis of gastric carcinoma cancer cells, induced by 5-Fu (a drug that is known to induce the apoptosis of cancer cells). The purpose of this study us to determine if Raman spectroscopy could be used to distinguish the apoptotic and non-apoptotic cancer cells and thus quantify the effect of the potential drug treatments. NIR Raman excitation was used in order to reduce the photochemical effect of the laser beam on the cancer cells. In addition, an area-scan excitation scheme was used to obtain the spatial-averaged Raman spectra of single cells under study so that nearly the whole cell's spectra were obtained for the apoptosis analysis. The advantage of the current excitation scheme is to reduce the data acquisition time needed in Raman mapping and to reduce the effect of spatial inhomogeneity of cellular components inside the cell's body on the analysis of Raman micro-spectroscopy, when the laser spot is much smaller than the size of the cells. We used conventional fluorescence microscopy to verify the apoptosis of the cancer cells after the treatment with 5-FU drug. We then measured Raman spectra of unstained apoptotic cells (after treatment with drug) and untreated cancer cells. The difference spectra were obtained to identify the effect of the drug treatment on the cancer cells. In addition, PCA multivariant statistics technique was used to distinguish the apoptotic cells and the control cells based on single cell's Raman spectra.

2. Materials and methods

2.1. Cell strains and chemical agents

The human gastric carcinoma cells (strain SGC-7901) used in this experiment were isogenic and purchased from Nanjing Keygen Biotech. Co., Ltd. (Nanjing, Jiangsu, China). 5-FU was purchased from Nantong Jinghua Pharmaceutical Co., Ltd. (Nantong, Jiangsu, China). Phosphate buffered saline (PBS) contains 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4 (pH 7.4). Hoechst 33258 dye was purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). New-born calf serum was purchased from GIBCO purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, Zhejiang, China).

2.2. Cell culture and drug treatment

Cells were initially cultured in growth medium RPMI-1640 (Hyclone), and then cells were transferred to a 6-well culture plate and incubated in the incubator at 37 °C and 5% CO_2. Three wells of cells were used as the control group without the treatment with 5-FU drug and the others as the treated group with 5-FU drug. After the cells were grown up to the 50% converge of the plates, 5-FU was added into the treated group to reach a final concentration of 25 μg/mL, while PBS of the same amount as 5-FU was added into the control group. Then both groups continued to be incubated in the incubator at 37 °C for 96 h. After inoculation, 0.25% trypsinase (Hyclone) were added into each group for hydrolyzation, respectively. The control and treated cells were then washed twice, rinsed, and immersed in PBS. The apoptosis of the treated cells was tested with dye Hoechst 33258. After the apoptosis was validated, the treated and control cells were used for the measurement of Raman spectra of single cells, respectively.

2.3. Experimental setup

The experimental setup of Raman tweezers is shown in Fig. 1. The system is a confocal device, as described previously [12–14]. A laser beam from a wavelength-stabilized diode laser (Hitachi, HL-7851G, 780 nm, 20 mW) was circularized with a pair of anamorphic prisms,

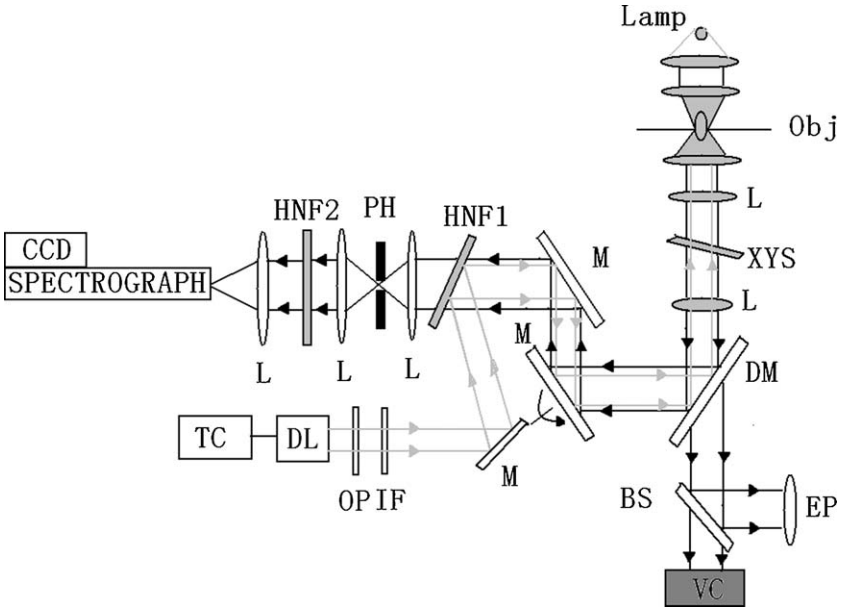

Fig. 1. Schematic of the experimental arrangement: TC, temperature controller; DL, diode laser; OP, optical isolator; IF, interference filter; M, mirror; L, lens; PH, pinhole; HNF1, HNF2, holographic notch filter; CCD, charge-coupled detector; DM, dichronic mirror; BS, beam splitter; Obj, objective lens; EP, eyepiece; VC, video camera; lamp, green-filtered xenon illumination lamp; and XYS, a pair of scanning mirrors in *x*- and *y*-axes.

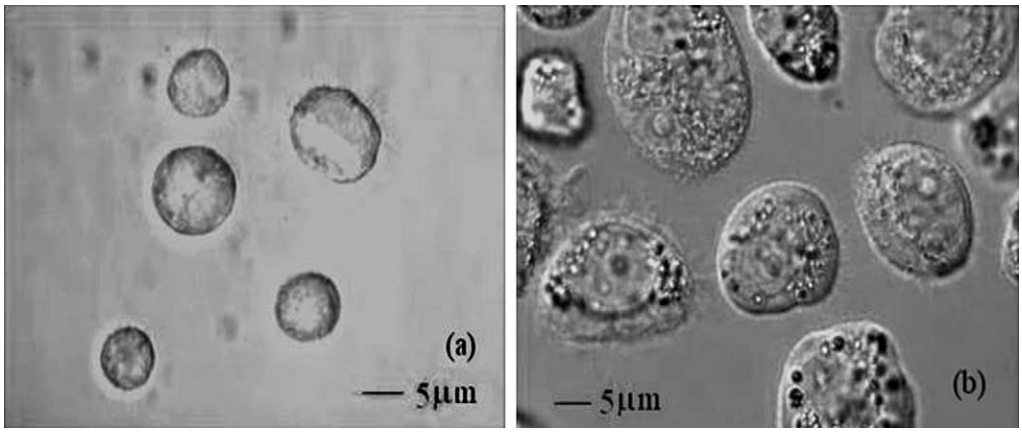

Fig. 2. Images of gastric carcinoma cells (a) and apoptotic cells (b), observed with a DIC microscope with an objective of 100×.

spatially filtered, and then introduced in an inverted differential interference contrast (DIC) microscope (Nikon TE2000-U) equipped with an objective (100×; numerical aperture of 1.30) to form a single-beam optical trap. The same laser is also used as the excitation source. Raman scattering light from a single cell was collected with the objective and then focused onto the entrance slit of an imaging spectrograph (Acton Research Co., SpectraPro2300i, 600 g/mm blazed at 750 nm) and recorded with a liquid-N_2-cooled charge-coupled detector (CCD, Roper Scientific Spec-10, Princeton Instruments). An interference filter (IF) was used to block the background emissions from the excitation source. A holographic notch filter (HNF) was used to eliminate the Rayleigh scattering and a confocal pinhole aperture (150 μm) was used to reject the off-focus light in order to increase the signal-to-noise ratio. A green-filtered illumination lamp and a video camera system were used to observe the image of the trapped cell during the acquisition of Raman spectra. Polystyrene beads (2 μm, Bands Laboratories) suspended in water were used for the system alignment and the calibration of relative Raman shifts to the excitation laser wavelength. The spectral resolution of our confocal micro-Raman system was estimated to be approximately 6 cm^{-1}.

In order to excite a large area of the cells, a pair of galvo-mirror scanners was used to steer the laser beam in both *x*- and *y*-directions, as shown in Fig. 1. A signal function generator (EE1642B, Nanjing Xinlian Telecommunication Instruments Co., Ltd., Nanjing, China) was used to provide electric voltages for scanning the mirrors. In the present experiment, the scan frequency was 8 Hz in the direction of the *x*-axis, and 0.3 Hz in the direction of the *y*-axis. The voltage of ~2.5 V was applied for both axes, which scanned the laser beam across an area of ~10 μm × 10 μm.

2.4. Raman spectral data acquisition and data analysis

The laser power was set as 15 mW and the Raman acquisition time was 15 s. These conditions were kept constant for all measurements. The test cell samples were kept in moist with PBS during data acquisition. Up to 20 cells of untreated cancer group and apoptotic group were measured. Raman spectra of individual cells were obtained through the scan-excitation mode. The background spectra of the solution were acquired with the same scan area without the cell.

In general, laser spot was about 1–2 μm in diameter and about 1–3.14 μm^2 in area [15]. However, the size of the gastric carcinoma and apoptotic cells were larger than 10 μm in diameter, as shown in Fig. 2. The area of laser spot was 1–4% of the surface area of single cells. In order to obtain spatial-averaged Raman spectral information of single cells, the laser spot was scanned across nearly the whole cell during Raman acquisition. The scanning area equals to the least square that just covers the whole cell.

Spectral process procedure of individual cells includes subtraction of background spectra, intensity calibration, 5-point smoothing, baseline calibration and normalization, and discrimination analysis was carried out using the software Micro Origin 8.0. PCA was carried out with the software Pychem 1.0.2 after

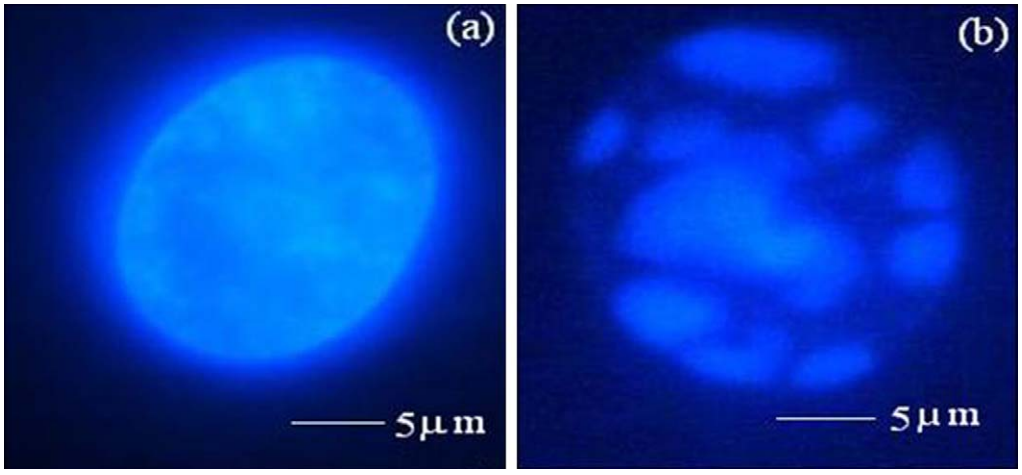

Fig. 3. Images analysis of the process of apoptosis: (a) a control cell, (b) a treated cell. Gastric carcinoma cells were stained with fluorescent dye Hoechst 33258. Apoptosis was induced by 5-FU. Nuclear morphological changes of gastric carcinoma cells during apoptosis were shown.

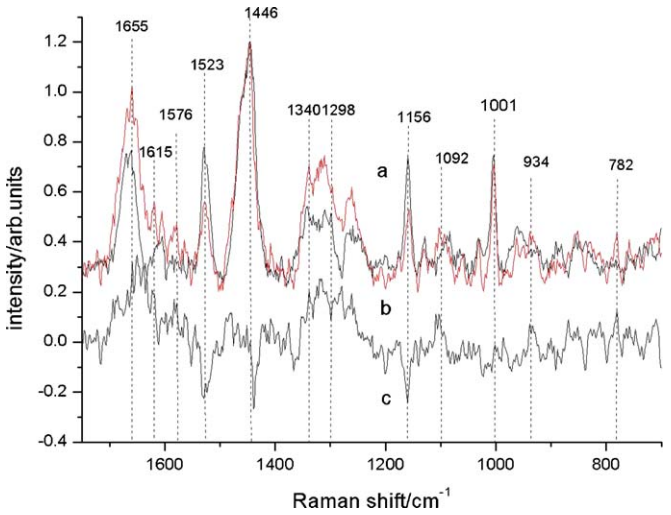

Fig. 4. Raman spectra of untreated gastric carcinoma cells (curve a) and apoptotic cells (curve b). Curve c was the difference spectrum between a and b. The position of Raman bands at 782, 934, 1001, 1092, 1156, 1298, 1340, 1446, 1523, 1576, 1615 and 1655 cm^{-1} were marked.

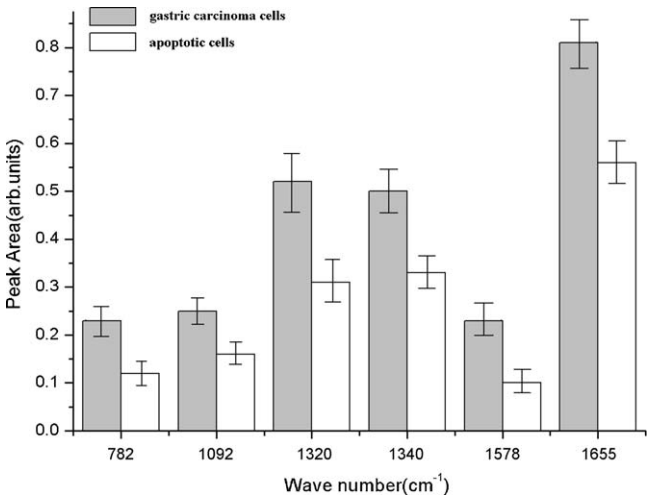

Fig. 5. Relative intensities of Raman peaks of single gastric carcinoma and apoptotic cells.

processing with the software Matlab 7.0, including 5-point average, intensity calibration, and normalization by area.

3. Results and discussion

3.1. Detection of apoptosis

The ability of a cell to undergo apoptosis is crucial during development, tissue homeostasis, and in the pathogenesis and treatment of disease [16–18]. To study the Raman spectra of apoptosis, it is important to validate apoptotic cells reliably. Here, we described a method to detect apoptosis based on the changes in chromatin and nucleolus distribution in the plasma membrane that occur during apoptosis. The control and treated cells were stained with Hoechst dye to test the process of the apoptosis. As shown in Fig. 3a, the nucleolus and chromatin of the control cell was intact, however, the chromatin of the treated cell was highly compacted and fragmented in Fig. 3b, which was in stage IIa as mentioned and regarded as an apoptotic cell.

3.2. Analysis of Raman spectra

The averaged Raman spectra of gastric carcinoma and apoptotic cells are shown in Fig. 4. The spectra of the control and apoptotic groups were averaged over 20 cells that were measured and the spectral heights were normalized to the height of 1446 cm^{-1}. The peak normalization eliminated the effect of variation of cell's size. The band 1446 cm^{-1} corresponds to CH_2 bending vibrations in the proteins and lipids of the cell. The peak assignments of the Raman peaks are given in Table 1.

The gastric cancer cells are composed of lipid, DNA and the associated carotenoid, proteins and amino acids, and their Raman spectra contained the similar characteristic Raman peaks [19] (Fig. 4). From the average spectra of gastric cancer, it was found that there were prominent spectral peaks at 782, 934, 1001, 1092, 1156, 1298, 1340, 1446,1523, 1576, 1615 and 1655 cm^{-1}. These spectra features arise from the molecular vibrations of gastric cancer components (carotenoids, lipids, nucleic acids and proteins) as summarized in Table 1 [19–22].

The apoptotic cells showed lower Raman intensities of nucleic acids, as indicated in the heights at 782, 1092, 1320, 1340, 1576 cm^{-1}, Besides the peaks mentioned above, the apoptotic cells also showed lower peak heights in the 1655 cm^{-1} band, which corresponds to the amid I bond of proteins. The reduction in the Raman bands observed suggests that the amount of nucleic acid, protein and lipid could be lower in apoptotic cells. This spectral finding was consistent with the fact that chromatin of the cell's nucleolus and other cellular components are highly fragmented in during apoptosis of the cancer cells (Fig. 5).

3.3. Analysis of Raman spectra by PCA

In order to determine if Raman spectra could be used to distinguish the apoptotic from control cells at the single cell level, we employed multivariate statistical tools for fine analysis of the spectral data. The major advantage of spectroscopic method is objectivity and the spectral data are amenable to multivariate statistical tools (e.g. PCA) [23]. PCA is a data regression method, by which large spectral data are reduced into small number of independent variables such as factors or principal components and contributions of these factors in a given spectra are known as scores [4]. Scores of factors is one of the parameters widely used for classification. In this study, we employed PCA to discriminate single control and apoptotic cancer cells.

Fig. 6 showed the scores of two principal components (PC1 and PC2) of 20 control cells and 20 treated cancer cells, based on their Raman spectra. It was found that the control cancer cells can be easily distinguished from the apoptotic cells by the PCA method. Furthermore, the scatter distribution of control cells was slightly narrower than that of the treated cells. The discrimination

Table 1
Raman bands observed in the spectra of human gastric carcinoma cells and their tentative assignments [19–22].

Peak position (cm^{-1})	Tentative peak assignments for Raman tissue spectra
781–782	Cytosine/uracil ring breathing (nucleotide) [19–21]
934	C–C stretch of proline ring and valine and protein backbone (α-helix conformation)/glycogen [20]
1001	Symmetric ring breathing mode of phenylalanine [19,20,22]
1092	O–P–O stretch (nucleic acids) [20,21]
1155–1156	C–C (& C–N) stretch of proteins (also caroteneoids) [20]
1298–1299	CH2 deformation mode of lipids [20]
1336–1340	Polynucleotide chain (DNA–purine bases) [21]
1446	CH2 bending mode of proteins [20]
1520–1538	–C═C– carotenoids [20]
1576–1579	Proteins [20,21]
1615–1616	C═C stretching mode of tyrosine and tryptophan [20]
1654–1655	Amide I (C═O stretching mode of proteins, α-helix conformation)/–C═C–lipid stretch [20,22]

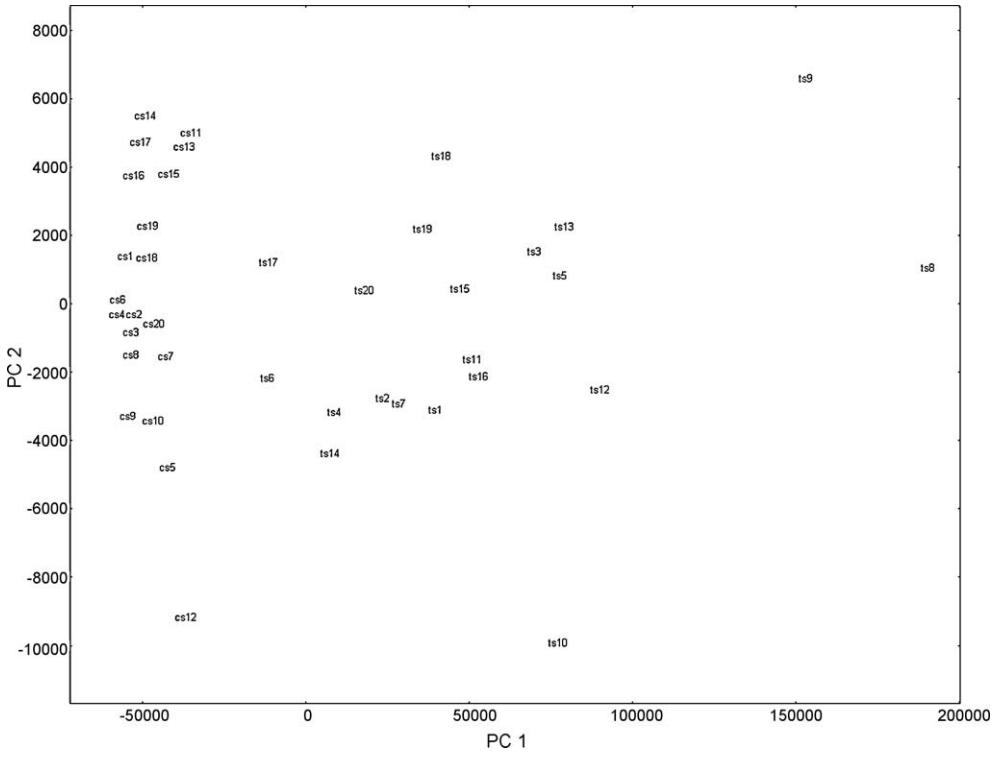

Fig. 6. PCA analysis of untreated (control) and treated gastric carcinoma cells based on single-cell Raman spectra. Twenty cells of the control group and treated group were analyzed. Cs: control group of human gastric carcinoma cells; ts: treated group of gastric carcinoma cells with 5-FU; number refers to the cell number measured.

achieved was mainly due to scores in PC1, which were negative for the control cancer cells and largely positive for apoptotic cells.

4. Conclusion

We have applied near-infrared Raman spectroscopy to analyze the apoptosis of single human gastric cancer cells, induced by 5-FU drug during incubation. The experiment showed that Raman spectroscopy is a sensitive technique for detecting the apoptosis of human gastric carcinoma cells. This analytical method could become a valuable tool in clinical application where there is a need for non-destructive real-time methods to detect the cure of the stomach cancer. Raman spectral difference between the untreated cancer cells and the apoptotic cells lies in a significant decrease in the nucleic acids peaks at 782 (45%) and 1092 cm^{-1} (35%), and peaks corresponding to nucleic acids and protein peak at 1320 (15%) and 1340 cm^{-1} (15%). The peaks corresponding to proteins are also drastically decreased by 56% at 1578 cm^{-1} and 32% at 1655 cm^{-1}. Based on PCA analysis, they can be classified into two separate parts mainly due to scores of PC1. The findings of these spectral signatures make it possible to use non-destructive Raman spectroscopy to monitor the apoptosis of stomach cancer cells and to test the effectiveness of the treatment of new drugs on the various cancer cells.

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