

Analysis of protein from nasopharyngeal carcinoma cell line CNE2 irradiated by X-ray using Raman spectroscopy

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Abstract. Our work aims to explore Raman spectroscopy (RS) to study the effects of total protein of nasopharyngeal carcinoma (NPC) cell line CNE2 treated by different doses of X-ray irradiation. The total protein is extracted from the cell line after irradiation with no incubation (0 h) and with incubation for 72 h, respectively. Both paired-samples *t* test and support vector machine (SVM) are employed for statistical analysis of Raman spectrum. The results show that several Raman bands assigned to total protein can be detected; and the X-ray irradiation have various effects on different components in total protein, especially for the dose higher than 6 Gy, and also associated with the incubation time after irradiation. The differences of content between 0 h groups and 72 h groups appear at bands of C–S stretching, C–H stretching, O=C–O[−] stretching and L-phenylalanine. It indicated no significant diversification on structures of protein, while the content may be varied during irradiation. Our spectroscopic results reveal that RS analysis of total protein of cell line irradiated by X-ray, in conjunction with mathematical statistical model, can be a potential method to explore the Raman characterization target of cell radiosensitivity, and then for making known the mechanism of radiosensitivity of tumor further.

Keywords: Nasopharyngeal carcinoma, Raman spectroscopy, support vector machine, total protein, X-ray irradiation

Abbreviations

NPC: nasopharyngeal carcinoma;

SVM: support vector machine;

RS: Raman spectroscopy.

1. Introduction

Radiotherapy is a primary treatment for nasopharyngeal carcinoma (NPC), and its efficacy is affected by the tumor in-house's radiosensitivity, host status and the radiation injury of normal tissue. Currently,

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the investigation on the changes of intracellular biomacromolecules due to X-ray irradiation effects has become one of main methods to study tumor radiosensitivity [8,15].

Raman spectroscopy (RS) is a laser-based type of spectroscopy that presents information on the chemical composition and structure of biological molecules and systems. As a method to acquire accurate frequency of molecular vibration, Raman spectroscopy allows noninvasive and nondestructive detection of changes of polypeptide and protein. RS discloses the fingerprint information about molecular vibrations which are significant to researchers [5,9,10,13].

Differences between normal and abnormal human cell lines (human breast epithelial cells, lymphocytes, NPC cells, etc. [13,20]) have been characterized from RS. The cell is made up of protein, nucleic acid, lipid and so on, as the base stock and functional unit, protein is involved in all metabolic processes, and thus Raman spectra of cell's protein after irradiation, the change of the molecular structure and content will be detected, which is helpful to explain the radiation injury mechanism. According to our knowledge, there are few reports on applying RS to detect the change of total protein of human NPC cell line treated by X-ray irradiation. We expect to acquire beneficial results to provide some significant references for relevant study through our work.

2. Materials and methods

2.1. Sample preparation

The human NPC cell line CNE2 (Fujian Provincial Tumor Hospital, Fuzhou, China) are cultured in RPMI-1640 medium supplemented with 100 U/ml streptomycin, 100 U/ml penicillin and 10% Newborn Calf Serum (Invitrogen Corp., USA). All the cells are grown in an incubator humidified 5% CO₂ atmosphere at 37°C. Anchored cells in a logarithmic growth phase are irradiated by X-ray with doses of 0, 2, 4, 6, 8 and 10 Gy, respectively, and in which no irradiation sample is to be as control, 15 cm × 15 cm of radiation field, and 100 cm of source-skin distance and 285 cGy/min of dose rate by the accelerator (SIEMENS Corporation, Germany). The samples of total protein are extracted from the cell line at two time points, which the one is after irradiation with no incubation (0 h) and the other is with incubation for 72 h, and with six dose groups for each time points, and Qproteome Mammalian protein Prep Kit (QIAGEN Corporation, Germany) to be used. The procedure suggested by the manufacturer is followed to make a cell's cracking mixture, weight drop, stewing, and then centrifuge to acquire the supernatant. Before the total protein samples are conserved at -20°C, the samples' concentrations were measured respectively using BioPhotometer 6131 (Eppendorf AG, Germany) and the correlative parameters have confirmed the purity of them.

2.2. RS of total protein

The total protein samples are deposited on aluminum flake and the Raman spectra are acquired using a Renishaw InVia Micro-Raman system with a 20× objective. 785 nm laser from a multi-element high power diode laser (about 20 mW of power) is used for excitation and the integration time is 20 s with 500–3200 cm⁻¹ spectral region.

2.3. Data analysis

Removed the baseline and background autofluorescence (Vancouver Raman Algorithm, BC Cancer Agency & University of British Columbia [11]). All the spectrum are averaged and then finally nor-

malized according to the spectral region. Then the SVM algorithm (supported by Libsvm2.84 [3]) is used to recognize the spectrum of every irradiated group and its control group. The training samples of each group are chosen at random and same number, and the rest to be recognized. According to C-SVM algorithm the automatic optimization parameter is utilized to predict the rest of corresponding samples.

3. Results

3.1. Spectral analysis

Figures 1 and 2 show the Raman spectra of total protein sample extracted from CNE2 cell line irradiated by different doses and following on with incubation of 0 and 72 h, respectively. There are several Raman bands assigned to total protein, such as 641 cm^{-1} assigned to C–S stretching and C–C twist of total protein, 952 cm^{-1} assigned to L-phenylalanine. Meanwhile the bands from leftover are also detected like 679 and 1014 cm^{-1} assigned to base G and DNA skeletal modes.

The band positions and the corresponding assignments are shown in Table 1 [1,2,6,7,9,12,14,16,18,19]. It can be seen that all of the spectral profile exhibits almost a similar with subtle differences, which indicates protein composition is not a significant change. However, there are varying intensities of corresponding bands between the irradiated and control groups, and statistically significant differences at 679 , 716 , 952 , 1420 , 2930 cm^{-1} band. The corresponding difference spectrum are shown in Figs 1(b) and 2(b), and focus on those visible variation of intensity in bands assigned to protein are discussed in detail below.

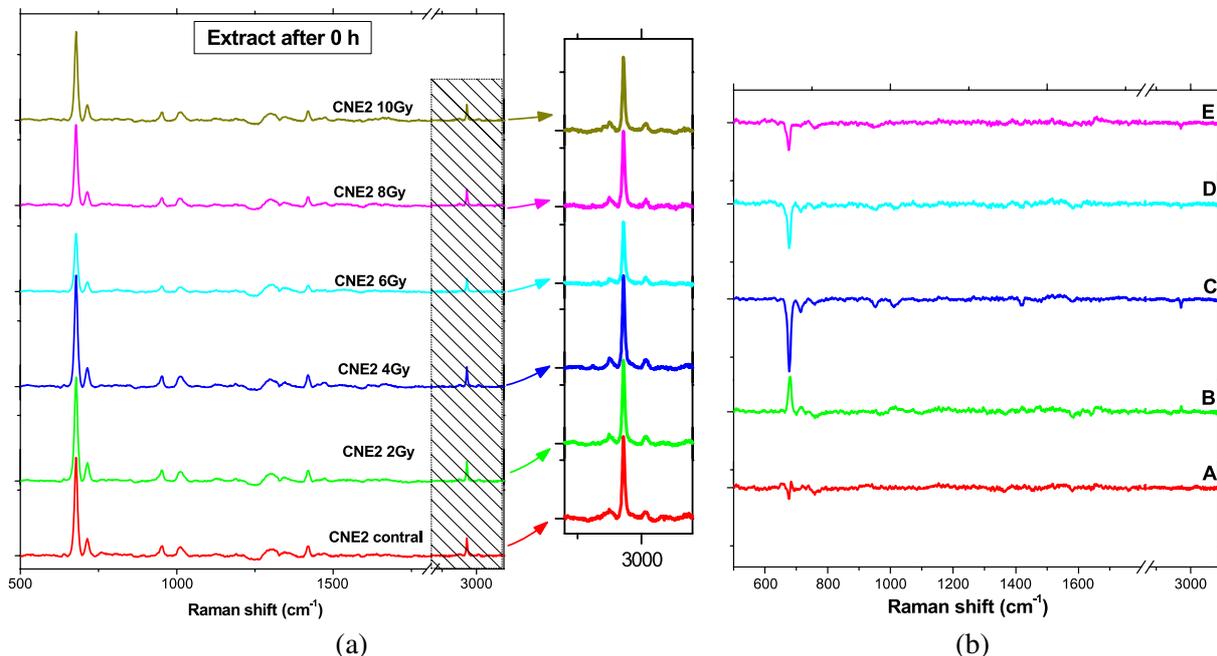


Fig. 1. (a) RS of total protein extracted from CNE2 cell line irradiated by five doses X-ray with 0 h incubation; and (b) the corresponding difference spectrum between irradiation group and control group: 2 Gy-control (A), 4 Gy-control (B), 6 Gy-control (C), 8 Gy-control (D), 10 Gy-control (E). (Colors are visible in the online version of the article; <http://dx.doi.org/10.3233/SPE-2012-0569>.)

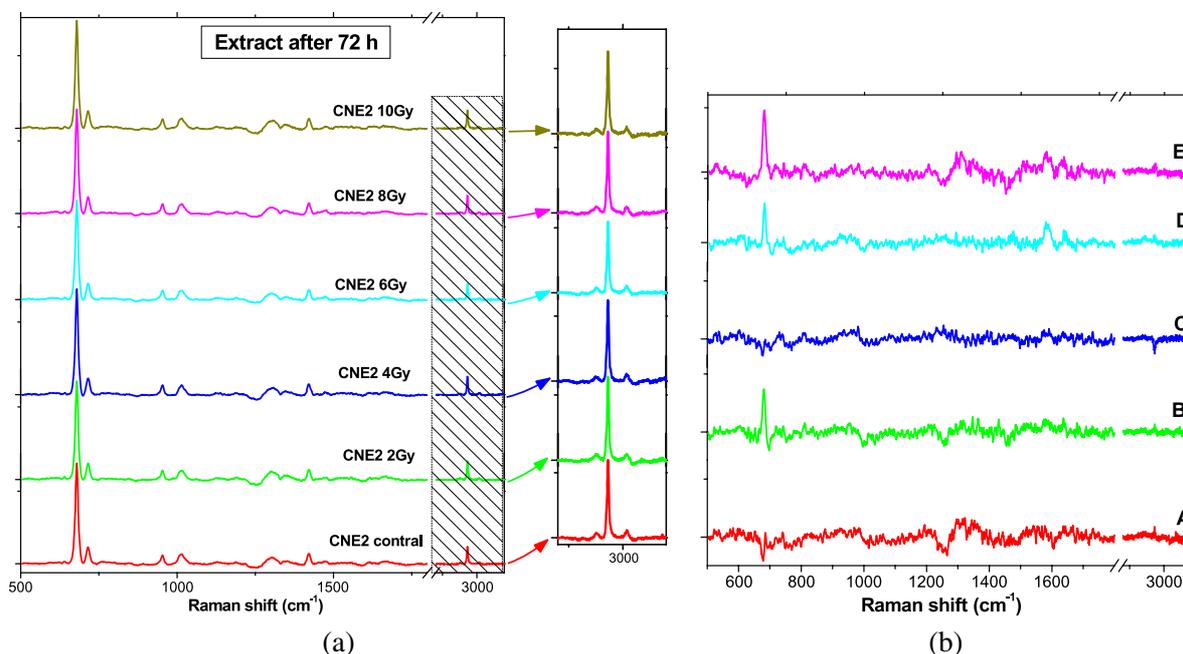


Fig. 2. (a) RS of total protein extracted from CNE2 cell line irradiated by five doses X-ray with 72 h incubation; and (b) the corresponding difference spectra between irradiation group and control group: 2 Gy-control (A), 4 Gy-control (B), 6 Gy-control (C), 8 Gy-control (D), 10 Gy-control (E). (Colors are visible in the online version of the article; <http://dx.doi.org/10.3233/SPE-2012-0569>.)

Table 1

Band assignments for Raman spectra of total protein extracted from CNE2 cell line irradiated by different doses X-ray

Control	Band (cm^{-1})					Assignments
	2 Gy	4 Gy	6 Gy	8 Gy	10 Gy	
641	641	641	642	640	640	C-S stretching, C-C twist, Tyrosine
679	679	679	679	679	679	G
717	717	716	716	716	716	C-S stretching
952	952	952	952	951	952	L-phenylalanine
1013	1014	1014	1012	1015	1014	DNA skeletal modes
1419	1420	1419	1419	1419	1419	Carboxylic acids: $\text{O}=\text{C}-\text{O}^-$ stretching
2879	2881	2879	2877	2874	2881	CH_2 stretching
2931	2931	2931	2931	2931	2931	CH stretching
3020	3018	3021	3021	3021	3018	C-H stretching (CH, CH_2 , CH_3)

- (1) At the position of 716 cm^{-1} assigned to C-S stretching, (1) for incubation 0 h, the intensity of 2 Gy irradiation group is nearly equal to control group (paired-samples t -test analysis, $p > 0.05$) while higher than control group at 4 Gy ($p < 0.05$); from 6 to 10 Gy group, the intensity decrease gradually with irradiation dose increases and all the intensity is lower than control group ($p < 0.05$). (2) for incubation 72 h, from 2 to 10 Gy group, the intensity increase gradually and is nearly equal to control group, the intensity at 10 Gy group is a little higher than control group, however, t test analysis does not confirm this difference ($p > 0.05$).

- (2) At the position of 952 cm^{-1} assigned to L-phenylalanine, (1) for incubation 0 h, the intensity of 4 Gy group is higher than control group ($p < 0.05$) while the others are a little lower than control group, however, t -test analysis does not confirm this difference ($p > 0.05$); (2) for incubation 72 h, from 2 to 10 Gy group, all the intensities are higher than control group ($p < 0.05$), and nearly equal to each other.
- (3) At the position of 1420 cm^{-1} assigned to $\text{O}=\text{C}-\text{O}^-$ stretching of carboxylic acids, (1) for 0 h, compared with the control group, the intensity of those radiation group is fluctuated significantly, in which 4 Gy group is higher than control group ($p < 0.05$) while lower case occurs at 8 and 10 Gy group ($p < 0.05$); (2) for 72 h, the intensity of 10 Gy is nearly equal to control group while higher case occurs at 6 and 8 Gy ($p < 0.05$).
- (4) At the position of 2930 cm^{-1} assigned to C-H stretching, (1) for 0 h, the intensity of 2 Gy group is nearly equal to control group ($p > 0.05$), and the intensity is higher than control group at 4 Gy group ($p < 0.05$) while lower from 6 to 10 Gy group ($p < 0.05$), as shown in the enlarged area of Fig. 1(a); (2) for 72 h, the intensity of 10 Gy group is nearly equal to control group while the others are higher than control group ($p < 0.05$), as shown in the enlarged area of Fig. 2(a).

All of these above may indicate there is no positive correlation with irradiation dose due to the changes of total protein. And comparing with control group at the mentioned bands above, 2 Gy group shows no significant difference; and the contents increase significantly in 4 Gy group, while the contents of C-S stretching, C-H stretching and $\text{O}=\text{C}-\text{O}^-$ stretching decrease significantly in 6 Gy group. Terms of the incubation for 72 h, the content of C-S stretching recover to basal level while L-phenylalanine increases significantly in all irradiated group; and the contents of C-H stretching and $\text{O}=\text{C}-\text{O}^-$ stretching increase when the dose is higher than 6 Gy.

3.2. Statistical analysis

Moreover, in order to statistically comparatively analyze the effects of total protein of CNE2 cell line irradiated by X-ray, SVM is utilized simultaneously to train and recognize for those significant changes. For 72 h incubation, the intensity in Raman spectra at 715 , 951 , 1419 and 2930 cm^{-1} , have been input into SVM algorithm, then 10 training samples are chosen at random and the rest to be recognized. The recognized accuracy (A) is defined by,

$$A = \frac{N_{\text{true}}}{N_{\text{total}}} = \frac{N_{\text{total}} - N_{\text{false}}}{N_{\text{total}}},$$

where N_{true} , N_{false} and N_{total} represent the number of rightly, wrongly and total classified samples, respectively. And the recognized results are shown in Fig. 3. The parameter A of 716 cm^{-1} is relatively lower with mean accuracy of 54.66%, but relatively higher at 951 and 2930 cm^{-1} , terms of the 8 Gy group reach to 83.33% and 80%, respectively. At 1419 cm^{-1} the mean accuracy is 75.33%, in which 10 Gy group is 79.76%. These show that L-phenylalanine, C-H stretching and $\text{O}=\text{C}-\text{O}^-$ stretching of CNE2 cell line irradiated by higher dose are statistically different to its normal status, and moreover the parameter A may indirectly reflect the differences of RS bands.

4. Discussion

Based on the aforementioned, it can be seen that the X-ray irradiation has various effects on different components in total protein, especially for the dose higher than 6 Gy, and also associated with the incu-

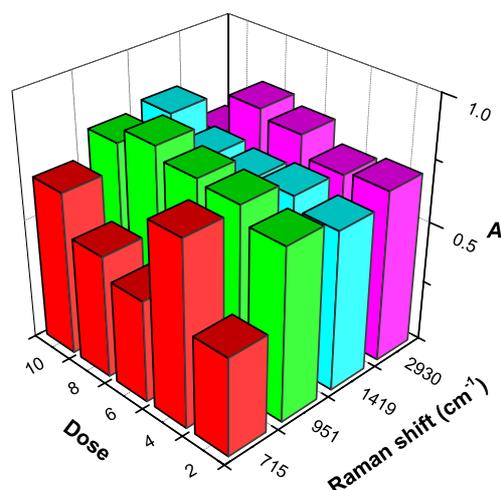


Fig. 3. The distribution of SVM recognition results at 715, 951, 1419 and 2930 cm^{-1} bands in different doses (2, 4, 6, 8 and 10 Gy). (Colors are visible in the online version of the article; <http://dx.doi.org/10.3233/SPE-2012-0569>.)

bation time after irradiation. In 72 h groups, contents of L-phenylalanine, C–H stretching and O=C–O⁻ stretching is increased while for C–S stretching is converted to original level. The probable reason is the cell damaged or death induced by the double strand breaking of DNA, meanwhile, in which DNA damage repair process is stimulated and its biological effects occurring after a few hours [4,17]. During the 72 h of incubation, those intracellular changes hand down by heredity in cells proliferation so as to affect the total protein synthesis, which led to change in elegant protein contents [19].

In addition, when X-ray irradiates cells, it acts on the water molecule by direct or indirect way, which leads to ionization and excitation of water molecule. Then reactive free radicals like OH⁻ and e⁻aq are formed to make damage of biologically active macromolecules such as nucleic acids, proteins, including enzymes, which cause them ionized, stimulated or chemical bond broken, and so given rise to change molecular structure and nature, and eventually result in anomaly and obstacle for metabolism. L-phenylalanine is a main structure of protein as one of the eight necessary amino acids, and plays an important role in cell metabolism. Affected by irradiation, the content change of L-phenylalanine is related to what kinds of proteins and biological function in cell, and what are the relationship with radiosensitivity and so on, all of these as we know, there is no report so far. Therefore, our present work may provide such molecule target or starting point to find relevant information for cellular radiosensitivity research. Moreover, the content change of the free carboxylic acid (O=C–O⁻ stretching) after irradiation may suggest the damaged degree of proteins or related biological macromolecules. Due to ionization break, sulfhydryl oxidation, double-sulphur bond reduction and side hydroxyl oxidation of peptide bond from protein or enzyme caused by irradiation, may lead to degradation and conformation change of protein. In view of this, the band of O=C–O⁻ stretching may be as one of a target to analyze cell's radiosensitivity which will be confirmed by further study.

5. Conclusions

In summary, the total protein's Raman spectral profile of CNE2 cell line irradiated by X-ray with the experimental dose are almost similar, which indicates no significant diversification in the components of

protein, and variation occur in the intensity of several bands related to irradiation dose and incubation time after irradiation. Taking into account the protein is the main executor of cell function, therefore, analysis to spectral changes of protein of cancer cell irradiated is useful to explore the Raman characterization target of cell radiosensitivity, and then for making known the mechanism of tumor radiosensitivity further.

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