Assessment of the radiotherapy effect for nasopharyngeal cancer using plasma surface-enhanced Raman spectroscopy technology

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Abstract: Nasopharyngeal cancer (NPC) is a malignant tumor of the head and neck, which is extremely sensitive to radiotherapy. The aim of this study is to evaluate the feasibility of a label-free nanobiosensor based on plasma surface-enhanced Raman spectroscopy (SERS) to assess the radiotherapy effect in NPC. Here, SERS measurements were performed on plasma samples from 40 pre-treatment and post-treatment NPC as well as 30 healthy volunteers. Results demonstrate that the spectral characteristic of post-treatment samples is obviously different from that of pre-treatment ones, owing to the changes of biomolecules in plasma induced by radiotherapy. Classification sensitivities of 83.3%, 61.8% and 95.1%, and specificities of 91.2%, 67.4% and 93% can be achieved for separating pre- and post-treatment samples, post-treatment and normal samples, and pre-treatment and normal samples, respectively, suggesting the great potential of plasma SERS method as a rapid and convenient tool for radiotherapy assessment and cancer screening in NPC.

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1. Introduction

Nasopharyngeal cancer (NPC) is a malignant neoplasm presenting a distinct racial and geographical distribution, with the highest incidence rate in the southeast area of Asia, including China (Guangdong, Hong Kong and Fujian), Malaysia, Indonesia and Singapore, which is 10-30 times higher than the western countries [1, 2]. Notably, NPC is often difficult to detect because of the non-specific nature of its clinical symptoms and the difficulty in visualizing the nasopharynx. When diagnosed, 70% of the patients have reached the advanced stage [3–5]. The treatment methods for NPC mainly include radiation therapy and chemotherapy, and a small number of cases are supplemented by surgery [6]. Because of intrinsically insidious anatomical location of NPC tumor, radiation therapy is the optimal choice in clinical practices [4, 6–10]. The main effect of radiotherapy is to induce the apoptosis of tumor cells via directly or indirectly damaging the cell's nucleic acid [11, 12]. As the development of radiation therapy, the 5-year survival rate of early NPC is more than 90% [13, 14]. Currently, various imaging technologies, such as magnetic resonance imaging (MRI), nasopharyngoscopy and computer tomography (CT) [15] are employed for evaluating curative effect of NPC after radiotherapy. While providing helpful information on the tumor region, tumor location, tumor size, region intensity, volume and degree, these technologies highly depend on the clinical experience of the clinician, and have inevitable subjectivity, which reduces the detection accuracy. Tissue biopsy followed by the histologic examination still remains the gold standard, however it is an invasive, complicated, time-consuming and subjective process, which is impractical for detection of high-risk patients having multiple suspicious lesions. As such, there is an urgent need to develop timely and noninvasive techniques for assessing the effect of radiotherapy in NPC.

Raman spectroscopy is a vibrational spectroscopic technique that can be used to optically probe the molecular changes associated with cancer status. However, the common Raman signals are extremely low and easy to be disturbed by fluorescence. In the past decades, the
advent of surface-enhanced Raman spectroscopy (SERS) significantly extends the application of Raman spectroscopy in the field of biomedical analysis, due to its extremely high Raman scattering efficiency achieved by the interaction between the nanostructured substrate and the analytes of interest. Many researchers have achieved some preliminary results using SERS for the detection of human samples, such as cell, tissue and body fluids [16–20]. For example, Lussier group designed a nanosensor created from borosilicate nanopipettes, which was used to locally monitor cellular secretion events near living cells [21]. Huang and his associates have proved that SERS spectroscopy is a promising analytical tool for investigation of biological tissue [22]. It has become an effective method for biochemical detection and analysis and has shown promising potential in cancer diagnostic application, especially in non-invasive body fluid analysis [23–26]. In clinical practice, analysis of blood is the most commonly used process for preliminary disease screening [27, 28]. Thus, several groups recently have developed a novel blood analytical technology based on SERS for cancer diagnosis [29–33]. This methodology has attracted much attention because plasma sample is easier to collect compared with other diagnostic samples such as cell and tissue, making it suitable for rapid and minimal invasive cancer diagnosis. Moreover, the subtle changes of biomolecules in plasma from cancer subjects can be sensitively explored by SERS spectrum due to that the characteristics of SERS peaks is dominated by various biomolecules. With the use of multivariate statistical algorithm, powerful diagnostic model can be built for identifying the cancer samples from normal ones, possibly achieving cancer screening.

In this work, the promising plasma SERS technology was further employed for the analysis of plasma samples from pre-treatment and post-treatment NPC patients, with the aim of investigating the feasibility of this method for radiotherapy assessment.

2. Experimental

2.1 Plasma samples

Plasma samples for this study were collected from 40 NPC patients before radiotherapy (pre-treatment subjects), the same patients after radiotherapy (post-treatment subjects) and 30 healthy volunteers provided by Fujian Tumor Hospital. After 12 hours of overnight, a single 5 ml peripheral blood sample was collected from the study subjects in the morning with the use of an anticoagulant. Then, blood cells were removed by centrifugation at 2500 rpm for 10 min to obtain the plasma. The plasma samples were kept at −80 °C immediately until use. This work was approved by the local ethics committee, and informed consents were obtained from all participating subjects.

2.2 Preparation for Ag nanoparticles

Ag colloids were produced using hydroxylamine hydrochloride and Ag nitrate, following the method reported by Leopold and Lendl [34]. Briefly, 9 ml of sodium hydroxide solution (0.1 M) were added to 10 ml hydroxylamine hydrochloride solution (0.06 M). Then the mixture was added immediately to 180 mL silver nitrate solution (0.0011 M). The mixture was stirred until a homogenous mixture was obtained. The concentration of silver nanoparticles is about $1.12 \times 10^9$ /ml. Finally, Ag colloid solution was centrifuged at 10 000 rpm for 10 min, and the final concentration after discarding a portion of the supernatant was used for plasma SERS measurement. Figure 1 showed the absorption spectrum of the silver colloid which represents an absorption maximum at 414 nm. And, a transmission electron microscopy (TEM) image of the prepared Ag nanoparticles was inserted in Fig. 1. The particle sizes follow a normal distribution with a mean diameter of 35 nm and standard deviation of 5 nm. And the SERS enhancement factor of Ag colloids is approximately $4.42 \times 10^6$. 
Fig. 1. The UV-vis absorption spectrum of Ag nanoparticles. The absorption maximum is located at 414 nm. The inserted picture shows the TEM micrograph of Ag nanoparticles.

2.3 SERS measurements

The SERS spectra of plasma samples were recorded using a confocal Raman microspectrometer (Renishaw, UK) under a 785 nm diode laser excitation. Each plasma sample was firstly mixed with Ag colloid at 1:1 proportion. Then a drop of the homogeneous mixture was transferred onto an aluminum plate for SERS measurement. The SERS scattering signals of plasma samples in the wavelength region of 400–1800 cm\(^{-1}\) were recorded within 10 s integration time with 20 × objective and 2 cm\(^{-1}\) spectral resolution. The incident power on the sample is approximately 2 mW. Spectral acquisition and analysis were performed using the software package WIRE 3.4 (Renishaw).

2.4 Spectral processing and analysis

First, the autofluorescence background signals were removed from the raw SERS spectra [35]. Then all background-subtracted SERS spectra were normalized to the area under curve in the 400–1800 cm\(^{-1}\) wavenumber range of each spectrum. A multivariate statistical analysis based on principal component analysis combined with linear discriminant analysis (PCA-LDA) was employed to explore potential diagnostic information and identify the three plasma groups. In brief, PCA was firstly used for reducing the high dimension of Raman spectral space into a few principal components (PCs) variables. Then, the most diagnostically significant PCs (p<0.05) were identified by using the independent-sample T test and fed into subsequent LDA for plasma samples classification. To further test the performance of the PCA-LDA-based diagnostic algorithm, the receiver operating characteristic (ROC) curves were generated by varying the different threshold levels with leave-one out cross-validation procedure [36]. The SPSS software package (SPSS Inc., Chicago) was used for above PCA-LDA analysis.

3. Results and discussion

To study the Ag colloid enhancement effects for plasma samples, we have recorded the Raman spectra and SERS spectra of plasma samples. Figures 2(a) and 2(b) show the SERS spectrum and regular Raman spectrum from the same plasma sample, demonstrating the significant enhancement of Raman signal achieved by SERS. This phenomenon can be attributed to the adsorption of biomolecules plasma on the surface of Ag nanoparticles,
leading to signal enhancement through localized surface plasmon resonance effect [37]. In addition, the signal of Ag nanoparticle (Fig. 2(c)) is extremely weak, thus has no effect on plasma SERS spectrum. Figures 3(a) and 3(b) represent the CT images of a pre- and post-treatment patient using radiotherapy. The corresponding plasma SERS spectrum belonging to above two statuses from the same patient are shown in Figs. 3(c) and 3(d). It can be found that the signals of plasma SERS vary when the tumor disappears induced by radiotherapy, which suggests the possibility of plasma SERS technology for predicting the effect of radiotherapy in NPC.

Fig. 2. Comparison of (a) SERS spectrum of the plasma sample from a patient with NPC acquired by mixing the plasma with Ag colloid at 1:1 proportion, (b) the regular Raman spectrum of the same plasma sample without the Ag NPs and (c) the background Raman signal of the Ag colloid. Instrument parameters (785 nm wavelength; 10 s acquisition time; 2 mW power).
Using Ag-NPs as substrate, we have successfully obtained plasma SERS spectra from 110 subjects. Figure 4(a) shows the normalized mean SERS spectra obtained from pre-treatment, post-treatment and normal group. The SERS peaks located at 492, 635, 724, 809, 883, 998, 1068, 1129, 1208, 1384, 1566 and 1646 cm$^{-1}$ can be clearly observed in all the three groups, with the strongest signals at 635, 1129 and 1566 cm$^{-1}$. Meanwhile, the significant Raman spectral differences also exist between three groups plasma samples. Particularly, the SERS bands at 527, 635, 809, 998, 1129, 1197 and 1566 cm$^{-1}$ are greater for the pre-treatment group than for normal one, while SERS peaks at 588, 725, 1068, 1327 and 1646 cm$^{-1}$ are lower in pre-treatment group. And, the spectral intensities of post-treatment group are between pre-treatment group and normal group at 492, 1068, 1372, 1646 cm$^{-1}$. Because SERS peaks are generated by biomolecules such as proteins, nucleic acids, and lipids, the variations in intensity of the SERS peaks could reflect biomolecular changes associated with cancer status.

To better understand the changes of biological constituents in plasma, prominent SERS bands were assigned tentatively [17, 38–47] (Table 1). Figure 4(b) shows the intensity of the plasma SERS spectrum exhibited decreased peaks at 1068 cm$^{-1}$ (proteins) in NPC subjects, indicating that patient plasma may be associated with a decreased concentration of these proteins. Lin et al. [46] also observed a decrease of proteins in nasopharyngeal cancer saliva by SERS. These results revealed that specific-protein changes between the NPC and normal groups could be detected by SERS, suggesting promising potential of plasma SERS for NPC screening. In addition, the SERS bands of L-arginine (492 cm$^{-1}$), Amide-VI (588 cm$^{-1}$), Tyrosine (635 cm$^{-1}$), D-galactosamine (883 cm$^{-1}$) in plasma of pre-treatment patients shows lower percentage signals than those of post-treatment plasma, suggesting an decrease in the percentage of amino acids and saccharides contents relative to the total SERS active components in plasma of NPC patients after radiotherapy [17]. The reason may be due to the
various metabolic status of pre- and post-treatment subjects as well as normal ones, which is in agreement with biochemical analysis results of colorectal cancer [42], cervical cancer [44] and gastric cancer [41].

In order to incorporate all significant SERS spectral features for radiotherapy assessment, PCA-LDA diagnostic algorithm was used for classifying the three groups. First, PCA was performed to reduce the originally spectral data dimensions to a few variates (PCs) which still remain most useful information. T-test on the PCs obtained by PCA showed that two PCs (PC 2 and PC 5) accounting for 23.5% of the variance were diagnostically significant (p <0.05) for discriminating pre-treatment samples from normal samples. Similarly, four PCs (PC 1-4; 57.7% the variance) and two PCs (PC 4 and PC 6; 13.4% the variance) were found to be the most diagnostically significant for discriminating post-treatment samples from pre-treatment group, and normal group, respectively. Then, the most significant PCs were fed into LDA algorithm to generate diagnostic model. Figures 5(a-c) show the discriminant results using posterior probabilities belonging to pre-treatment, post-treatment and normal group as calculated by the LDA.
The present study showed that the diagnostic sensitivity and specificity of 83.3% and 91.2% for identifying pre- from post-treatment samples can be achieved using the PCA-LDA model. And, the pre-treatment samples can be identified from the normal ones with the diagnostic sensitivity and specificity of 95.1% and 93.0%. Meanwhile, only 61.8% of sensitivity and 67.4% of specificity can be obtained for discriminating between post-treatment and normal samples. These statistical results are summarized in Table 2 and can be explained. Due to the biological effects of radiation, the cancer cells can be killed and the growth of them will be stopped at the same time, leading to a reduced tumor size or even disappearance of tumor. Thus, the amount of metabolites in the peripheral plasma released by tumor cells will significantly decrease after radiotherapy [12]. Owing to these changes in biological molecules, the post- and pre-treatment groups can be well discriminated by characterizing the plasma SERS spectra and comprehensively exploring the diagnostic information via PCA-LDA. Meanwhile, the discriminant results for post-treatment and normal groups are poor, which can be attributed to that the post-treatment patients without distant metastasis have similar physiological status to the normal ones, especially for peripheral plasma. These results demonstrated that we might achieve the radiotherapy assessment by comparing the post-treatment group with pre-treatment and normal group, respectively. In addition, this novel plasma analysis method based on SERS also presented wonderful diagnostic efficiency for discriminating cancer ones from the normal. This diagnostic capability has been shown in our previous work [17].

To compare the classified performances for the three groups, receiver operating characteristic (ROC) curves were generated (Fig. 5(d)) from the scatter plots at different threshold levels. The integration area under the ROC curve is 0.933 for identifying pre-treatment and post-treatment, which is higher than post-treatment vs. normal group (0.704). Moreover, excellent diagnostic performance for cancer and normal groups is further confirmed by the integration area of 0.982.
Compared with traditional methods such as CT, MRI and biopsy for diagnosis and therapy monitoring, the proposed technology based on plasma SERS have numerous advantages. First, it is a minimal invasive and simple procedure and a rapid and continuous assessment can be achieved. Thus, it may provide the clinician with important, dynamic and objective information of radiotherapy response from patients, which is vital for the clinician to adjust the subsequent radiotherapy strategy. Second, owing to the ability of SERS for plasma detection at molecular level, it is possible to use plasma SERS for early screening of NPC. Besides, prognosis prediction after radiotherapy is another challenge in clinical practice. We believe this novel technology is promising for this goal with the exploration of reliable biomarkers in the future.

Table 2. Classification results of plasma SERS prediction of the three groups using PCA-LDA method

| Diagnostic combinations | Predicted parameters | Sensitivity (%) | Specificity (%) |
|-------------------------|----------------------|----------------|-----------------|
| Pre-treatment VS. Post-treatment | 83.3                  | 91.2            |
| Post-treatment VS. Normal | 61.8                  | 67.4            |
| Pre-treatment VS. Normal | 95.1                  | 93.0            |

4. Conclusion

We have developed a highly sensitive methodology which uses silver nanoparticle based surface-enhanced Raman spectroscopy to analyze the plasma samples from pre-treatment, post-treatment NPC patients and healthy volunteers. Results shows that there are obvious...
spectral differences (peak intensity) between pre- and post-treatment subjects, and high classification accuracy can be achieved using the PCA-LDA diagnostic algorithm, demonstrating the great potential of plasma-SERS analysis combined with PCA-LDA diagnostic algorithms for radiotherapy assessment and cancer screening in NPC. Our next step is to use this method for predicting the 5-years survival rate of patients after radiotherapy through distinct SERS spectral characteristic of plasma samples.

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**Disclosures**

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