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Research Article

Discrimination of Nasopharyngeal Carcinoma from Noncancerous *Ex Vivo* Tissue Using Reflectance Spectroscopy

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Reflectance spectroscopy is a low-cost, nondestructive, and noninvasive method for detection of neoplastic lesions of mucosal tissue. This study aims to evaluate the capability of reflectance spectroscopy system under white light (400–700 nm) with a multivariate statistical analysis for distinguishing nasopharyngeal carcinoma (NPC) from nasopharyngeal benign *ex vivo* tissues. High quality reflectance spectra were acquired from nasopharyngeal *ex vivo* tissues belonging to 18 noncancerous and 19 cancerous subjects, and the combination of principal component analysis-linear discriminant analysis (PCA-LDA) along with leave-one-spectrum-out cross-validation (LOOCV) diagnostic algorithm was subsequently employed to classify different types of tissue group, achieving a diagnostic sensitivity of 73.7% and a specificity of 72.2%. Furthermore, in order to distinguish NPC from nasopharyngeal benign *ex vivo* tissues based on reflectance spectra simply, spectral intensity ratios of oxyhemoglobin ($R540/R576$) were used as an indicator of the carcinogenesis associated transformation in the hemoglobin oxygenation. This tentative work demonstrated the potential of reflectance spectroscopy for NPC detection using *ex vivo* tissue and has significant experimental and clinical value for further *in vivo* NPC detection in the future.

1. Introduction

Nasopharyngeal carcinoma (NPC) is a prominent malignant tumor that occurs in the top and the side wall of nasopharyngeal cavity with no obvious early symptom, which directly causes harm to human health [1]. The incidence of NPC has remained high in southeast Asia, northern Africa, parts of the Middle East, and so forth. We should be concerned about the fact that approximately 80% of NPC cases were reported from China, especially in southern provinces, such as Guangdong and Fujian [2–4]. NPC was defined as a special disease with a remarkable racial and geographical distribution worldwide [5]. According to the epidemiological investigation, NPC has various factors to its etiology, and the main relevant risk factors include inherited genetic factors, the dormant infection of Epstein-Barr virus (EBV), and natural environment factor [6]. The clinical reports indicated that NPC patients treated in Stages I and Stages II have 5-year survival rates of approximately 90% and 75%, respectively. However, early stage of NPC has no obvious symptoms until the middle and advanced stages of disease [2, 6, 7]. Therefore, early diagnosis is the effective way to improve the survival rates of patient and promote the quality of their life.

Currently common detection and screening method for NPC is dependent on clinicians' experience, which easily causes possible diagnostic errors owing to lack of the biochemical information of suspicious lesions. Although the histopathological examination of biopsy is still the golden standard for NPC detection [8], it involves the destruction and invasion of tissue from suspected lesions. Diagnostic

techniques based on optical spectroscopy, in contrast, can provide quantitative information in real time from tissues, which are excellent in the field of precancerous tumor localization and pathology analysis.

Reflectance spectroscopy has attracted wide attention in human tissue study including oral, breast, lung, bladder, cervical, esophagus, kidney, and prostate tissue [9–14], due to its simple operation and low investment cost of equipment. In reflectance, broadband beam interaction with the biological tissue surface and two forms of cases can occur, that is, multiple elastic scattering and absorption phenomenon [15]. In this process, part of broadband beam returns as reflectance spectral signals, which are able to provide the optical properties of the tissue [16]. The reflectance spectral signatures are dependent on biological tissue morphology, surface characteristics, and structural components [13, 15]. Hence, reflectance spectra have certain advantages for explaining major tissue features within early cancerous transform, for example, the cellular metabolism, capillary distribution, and hemoglobin oxygenation [15, 17–19].

Zonios et al. reported a diffuse reflectance spectra study on human adenomatous colon polyps, showing that differences can be ascribed to hemoglobin concentration and effective scatterer size between normal and adenomatous tissue sites [15]. Utzinger and coworkers have investigated an exploratory pilot study to measure reflectance spectra at different source-detector separations from normal and neoplastic ovarian tissue and found the changes of intensities of the reflectance spectrum between 550 and 580 nm, which are dramatically attributed to the absorption of blood oxygenation [20]. Subhash and coworkers have characterized reflectance spectral intensity ratio of oxygenated hemoglobin absorption bands ($R540/R570$) associated with different types of lesions and histopathological conditions, and results showed that the $R540/R570$ ratio for malignant lesions was always lower than that for normal areas [13]. Li et al. used the reflectance ratio $R540/R575$ method to distinguish malignant from nonmalignant sites in human *ex vivo* gastric epithelial tissues and found some pronounced differences in various pathological types [21]. Zhao et al. reported a study about oxygenated hemoglobin absorption band ratios of esophageal epithelial tissues at different heat treatment temperature, showing the capability of employing oxygenated hemoglobin band ratios $R540/R570$ as indicators of malignancy [22]. Recent results have shown that tissue absorption in the short wavelength region and visible region is assigned to hemoglobin, with the oxygenation forms exhibiting different absorption spectral characteristic [23]. These reports demonstrated that reflectance spectroscopy could obtain spectral information about the pathological changes of tissue, which can serve as important supplement information for clinical diagnosis. Nevertheless, as far as we are concerned, still few reports have been found using reflectance spectroscopy for NPC detection.

In this preliminary study, we investigated reflectance spectral signals from human nasopharyngeal *ex vivo* tissue with different pathological types. Reflectance spectra were subjected to multivariate statistical tools, including principal component analysis-linear discriminant analysis (PCA-LDA) algorithm together with the leave-one-spectrum-out cross-validation (LOOCV). To validate the feasibility of reflectance spectroscopy as a tool to differentiate noncancerous *ex vivo* tissues from NPC, the receiver operating characteristic (ROC) curve was employed. Empirical method using reflectance spectral intensity ratios of oxyhemoglobin bands ($R540/R576$) was used to distinguish cancer from benign *ex vivo* tissue.

2. Materials and Methods

2.1. Patients and Sample Preparations. Nasopharyngeal *ex vivo* tissues for this study were collected from 37 patients who were recruited for biopsy from nasopharyngeal cavity lesions with written informed consent. This procedure was authorized by the Ethics Committee of Affiliated Fuzhou First Hospital of Fujian Medical University. All nasopharyngeal tissues were divided into two groups with clinical diagnosis (19 NPC samples and 18 benign tissue samples), as shown in Table 1. The pathological types of NPC patients contain 17 undifferentiated nonkeratinizing carcinomas and 2 differentiated nonkeratinizing carcinomas. The pathological types of benign tissues consist of 15 chronic inflammations, 2 lymphomas, and 1 lymphoid tissues hyperplasia. These fresh tissue samples were immediately stored at 4°C until being sent to the laboratory moments later, where they were stored in a minus 80°C refrigerator until experimental measurements. These targets were placed on an aluminum plate and thawed at room temperature immediately before spectral measurement (Figure 1).

2.2. Laboratory Instruments. All the reflectance spectra of nasopharyngeal *ex vivo* tissues were recorded using a special measurement system (Verisante Technology, Inc., Vancouver, Canada); more details were presented in previous report [24]. In brief, this detection system included a light source (a xenon arc lamp), a spectrometer (USB2000, Ocean Optics), and a PC. A band-pass filter (400–700 nm) was installed at the reflectance spectrograph entrance to obstruct the reflected unwanted light to improve reflectance signals detection. The exposure time of the spectrometer is 200 ms. An optical fiber is used to collect reflectance spectra, of which the entrance corresponds to a spot size of 1 mm diameter on the sample. The dimensions of the samples are generally between 1.8 and 2.5 mm. Three reflectance spectra were obtained from different positions for each *ex vivo* tissue and averaged to a mean spectrum so that we could acquire representative spectra for each sample. This detection system was calibrated by a certified reflectance standard (WS-1-SL, Labsphere) before each measurement to eliminate interference by the status of the system.

2.3. Data Processing and Analysis. To eliminate the interference of slow-moving backgrounds, the raw spectra were smoothed by using Savitzky-Golay method with points of window of 50 and polynomial order of 5. Reflectance spectra in the 400–700 nm range were subjected to principal component analysis (PCA) and linear discriminant analysis (LDA) combined with LOOCV by using SPSS statistical software

Table 1: Sample characteristic.

Sample type	Pathologic types	Number
Nasopharyngeal cancerous (NPC) patients ($n = 19$)	Undifferentiated nonkeratinizing carcinomas	17
	Differentiated nonkeratinizing carcinomas	2
Nasopharyngeal noncancerous patients ($n = 18$)	Chronic inflammations	15
	Lymphoma	2
	Lymphoid tissues hyperplasia	1

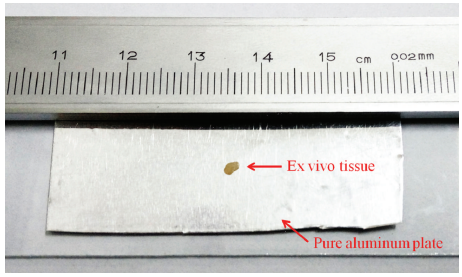

Figure 1: Nasopharyngeal *ex vivo* tissue placed on the pure aluminum plate. Pure aluminum and sample are pointed out by the black arrow.

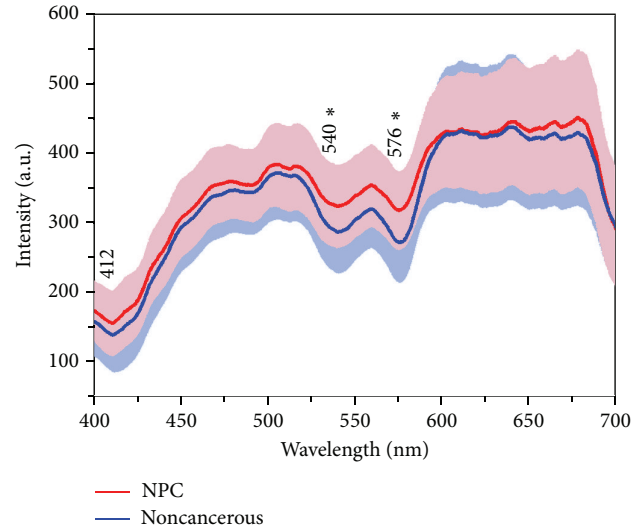

Figure 2: Mean reflectance spectra from NPC (red line, $n = 19$) and noncancerous *ex vivo* tissue (blue line, $n = 18$). Asterisks indicated significant difference (p value < 0.05) after t-test, in which the p values are 0.006 and 1.6×10^{-4} for 540 nm and 576 nm, respectively. Areas (shaded) illustrate the respective standard deviations of the means spectra.

(SPSS Inc., Chicago). PCA-LDA was employed to achieve a diagnostic algorithm for classification between different groups within the dataset. Briefly, PCA is a statistical method that is used on the dataset to reduce the dimensions of the data, while finding a series of the linear combination of the original variables, so-called principal components (PCs) [25, 26]. An independent-samples t-test was used to determine significant PCs (p value < 0.05) [25, 27]. The findings, which represent most of the significant variance in the dataset, are selected as input data for the development of LDA model for tissue classification [28]. LDA is a supervision algorithm that maximizes the covariance in the data between different groups and minimizes the variances within group [27]. The classification efficiency of the PCA-LDA diagnostic models for correctly predicting the sample groups (i.e., normal versus malignancy) was evaluated by LOOCV method so that to eliminate the risk of overfitting [29, 30]. The receiver operating characteristic (ROC) curves were plotted to evaluate the performance of the above algorithm [26].

3. Results

3.1. Analysis of Reflectance Spectra. Figure 2 shows the comparison of mean *ex vivo* reflectance spectra with standard deviations (SD) of the biopsy lesions from malignant sites and benign mucosa sites with the same detection system setup. Prominent valleys of reflectance spectra were observed in 412 nm, 540 nm, and 576 nm which can be attributed to absorption from hemoglobin bands [31, 32]. For all the spectra measured, the absorption peak intensity from NPC group was slightly weaker than that of noncancerous group in these absorption valleys. Notable absorption variations in reflectance intensity are more significant at green light region (around 540 nm and 576 nm bands), indicating an increase of microvasculature and hemoglobin [12, 13]. Significant differences were found in the spectral valleys at 540 nm (p value $= 0.006 < 0.05$) and 576 nm (p value $= 1.6 \times 10^{-4} < 0.05$) between the two groups by using the analysis of t-test.

3.2. Multivariate Statistical Analysis. Multivariate analyses including PCA and LDA were explored to evaluate the feasibility of classifying cancerous samples from noncancerous ones by employing the recorded reflectance dataset. Figure 3 shows this multivariate statistical analysis in a flowchart. In the first step the dataset underwent dimension reduction using unsupervised PCA to extract the principal components (PCs) which represent the measured spectra by a weighted linear combination. The PCs were tested by independent-samples t-test. PC3 and PC6 have the significant variance in the dataset (p value < 0.05) and were selected to discriminate the two pathological groups, in which the p values of are 0.022 and 0.007 for PC3 and PC6, respectively. The selected PCs (PC3 and PC6) were subsequently imported into LDA model with LOOCV method to evaluate classification efficiency.

The scatter plot of the diagnostically significant PC3 versus PC6 scores is displayed in Figure 4. Also displayed is the diagnosis equation ($0.738\ \mathrm{PC3} + 0.836\ \mathrm{PC6} + 0.017 = 0$) that separates the two groups of *ex vivo* tissue according to the result of statistical analysis. The diagnosis equation is defined as $Z = aX + bY$, where a and b are the standardized canonical discriminant function coefficients of PC3 and PC6 (0.738 and 0.836, resp.). This decision line is controlled by choosing the coordinate parameters of X and Y. Figure 5 shows the posterior probability generated from PCA-LDA algorithm belonging to noncancerous and NPC subjects, with a diagnostic sensitivity of 73.7% (14/19) and a specificity of

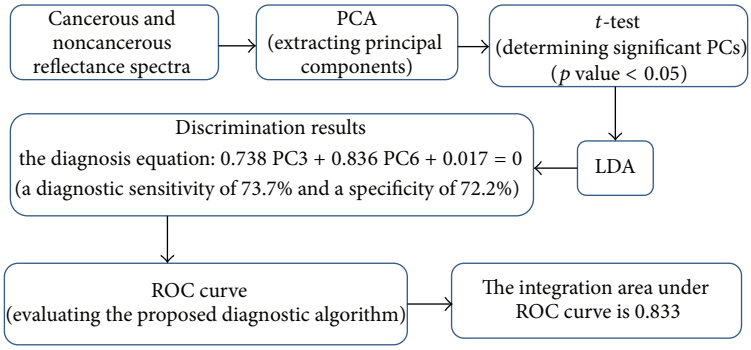

Figure 3: The work flowchart of multivariate statistical analysis.

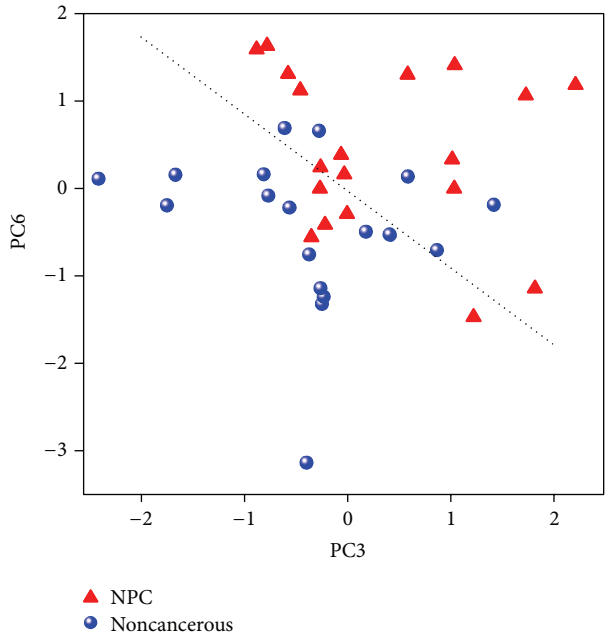

Figure 4: A two-dimension scattered point diagram of the PCA result for NPC group (red triangles ▲) and noncancerous group (blue circle ●). The dotted lines (0.738 PC3 + 0.836 PC6 + 0.017 = 0) served as a diagnostic method for NPC sample and noncancerous sample differentiation.

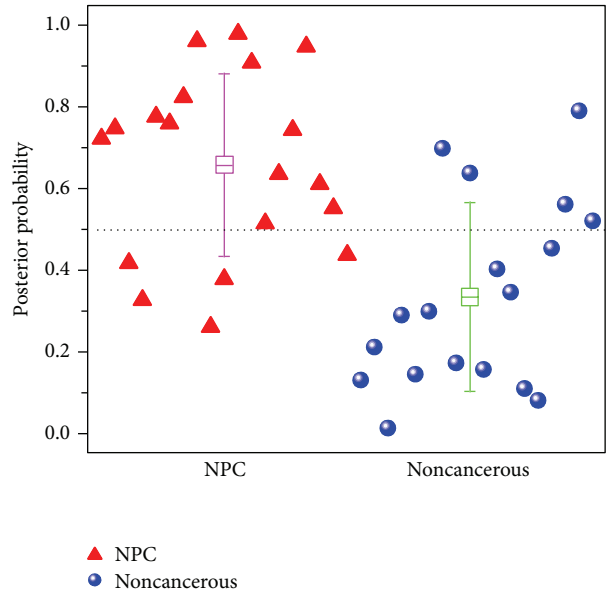

Figure 5: Scatter plots of the posterior probability belonging to NPC and nasopharyngeal noncancerous subjects (▲—NPC group; ●—noncancerous group), generated from PCA-LDA diagnosis algorithms along with LOOCV method. Error bars represent the mean and standard deviation of NPC (magenta) and nasopharyngeal noncancerous (green) *ex vivo* tissue posterior probability. The dotted lines represented the sensitivities and specificities for separating NPC from nasopharyngeal noncancerous tissue.

72.2% (13/18). The dotted lines were set to 0.5, where the total number of misclassified tissues was minimized [33], which represented the sensitivities and specificities for separating NPC from nasopharyngeal noncancerous *ex vivo* tissue. The sensitivity and specificity were defined as [34]

$$\text{Sensitivity} = \frac{\text{True Positives}}{\text{True Positives} + \text{False Negatives}},$$
$$\text{Specificity} = \frac{\text{True Negatives}}{\text{True Negatives} + \text{False Positives}}. \quad (1)$$

Figure 6 is the ROC curve, which was generated from the posterior probability plot at different threshold levels to evaluate the proposed diagnostic algorithm. The integration area under ROC curve (AUC) is 0.833, which further demonstrates that using reflectance spectra with multivariate statistical algorithms may be viable for discriminating NPC group from benign *ex vivo* tissue group.

3.3. Analysis of Hemoglobin Absorption Ratio R540/R576. Significant differences were found in the spectral valleys at 540 nm (p value = 0.006 < 0.05) and 576 nm (p value = 1.6×10^{-4} < 0.05) between the two groups by using the analysis of t-test. The ratios of intensity of 540 nm and 576 nm were calculated for each original reflectance spectrum. Then all the ratios were examined by t-test, and the p value is 1.89×10^{-7}. The ratios of $R540/R576$ were calculated by the related original data, and then these original ratios were averaged.

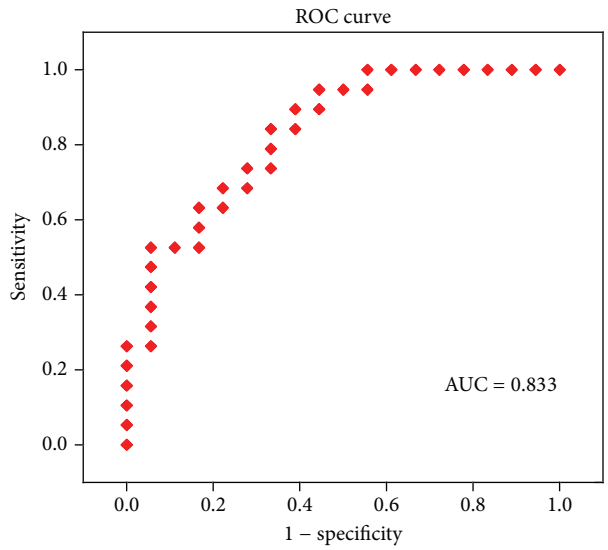

Figure 6: ROC curve for discriminating NPC from nasopharyngeal noncancerous *ex vivo* tissue for the training dataset by employing PCA-LDA diagnosis algorithms combined with LOOCV method. The AUC is 0.833.

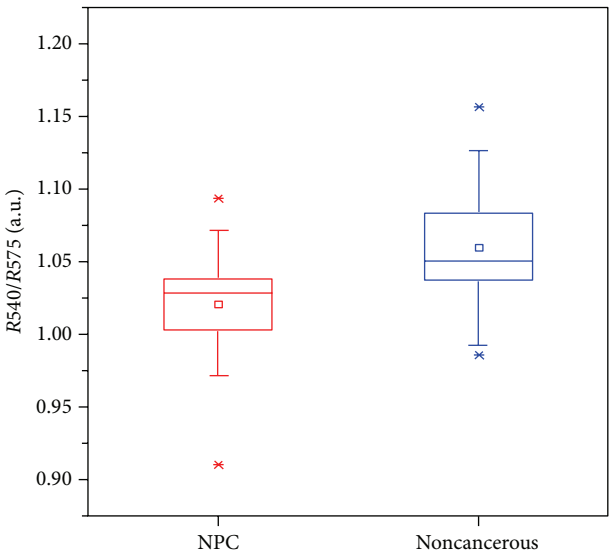

Figure 7: Mean reflectance ratios ($R540/R576$) of NPC (red box) versus those of noncancerous group (blue box).

Finally, the mean ratios were used to draw the boxplots (Figure 7). Figure 7 shows the comparison of intensities and standard deviations of the mean $R540/R576$ ratios between nasopharyngeal noncancerous (blue box) and NPC (red box) *ex vivo* tissues. In the case of noncancerous tissue, the mean $R540/R576$ ratio was found to be higher than that of nasopharyngeal malignancy group (1.06 versus 1.02).

4. Discussion

In this work, we investigated reflectance spectra obtained from benign and malignant *ex vivo* tissues in the human nasopharynx. The results of this tentative study show that there were some differences in reflectance spectra of *ex vivo* tissue between NPC and noncancerous subjects, demonstrating potential of reflectance spectroscopy for NPC detection and preliminary precancer screening. Our spectral measurement (Figure 2) shows that reflectance intensity curves of both *ex vivo* benign and malignant mucosa tissues are similar at 400–700 nm. However, the differences of reflectance intensity exist in curve valleys at around 412, 540, and 576 nm region owing to the absorption from oxyhemoglobin bands [31, 32]. These spectral absorption valleys are much more significant in the malignant tumor spectrum at 500–600 nm bands. The spectral absorption characteristics at 540 nm and 576 nm in *ex vivo* nasopharyngeal mucosa may arise from the absorption from oxyhemoglobin associated with high demand of oxygen by cancerous tissues, leading to a decrease in the content of oxygen levels in the blood [13, 23]. Therefore, the spectral absorption of cancerous group is weaker than that of noncancerous group at 540 nm and 576 nm (Figure 2). In the case of malignant lesion tissues, reflectance spectral intensities at 412 nm, 540 nm, and 576 nm bands were slightly stronger than that of noncancerous tissues. One possible explanation for the special spectral intensity differences is that cancerous tissues are often characterized by abnormally histological structure and microvascular volume [12, 13, 15]. In addition, collagen is more densely distributed in the submucosa [15], which is usually able to affect reflectance property of tissue surface.

These preliminary results may indicate the carcinogenesis process of nasopharyngeal carcinoma, since the cancer cells' growth and proliferation relate intimately on the capillary proliferation and nutrient requirements, which caused the metabolic disorders during this period. The tissular capillary will continue to spread, which may lead to the anomalies of histological structure. Furthermore, the cancerous tissue clonal expansion and evolution cause a significant decrease in oxygenated hemoglobin in tumor tissue during the long process of malignant cancer [13, 15, 35]. So, malignant tissues are of low blood-oxygen content owing to metabolic disorders [15]; that is, oxygenated hemoglobin content will be greatly reduced in cancerous tissues.

To further evaluate the capability of reflectance spectroscopy for differentiating nasopharyngeal malignant tumor from benign *ex vivo* tissue, multivariate statistical analysis (PCA-LDA) was performed on the spectral analysis. PCA-LDA yields a sensitivity of 73.7% (14/19) and a specificity of 72.2% (13/18), which demonstrates the availability of the diagnostic algorithm for distinguishing NPC from noncancerous tissue samples by using reflectance spectra.

At the same time, we used a simple empirical method; that is, reflectance spectral intensity ratios ($R540/R576$) were used as an indicator of neoplastic associated changes in the hemoglobin oxygenation. The mean intensity of $R540/R576$ ratio of oxyhemoglobin for malignant and benign tissue was 1.02 and 1.06, respectively. This computation coincides with the results of Subhash et al. [13]. The mean reflectance spectral intensity ratio ($R540/R576$) of oxyhemoglobin was weaker for NPC compared to noncancerous *ex vivo* tissue, which is probably for the reason that malignant tissues are much hungry for oxygen.

Finally, we have compared the diagnostic result of the *R*540/*R*576 method with that of the PCA-LDA algorithm. The *R*540/*R*576 method generated a sensitivity of 78.9% and specificity of 69.8% for differentiation between *ex vivo* malignant and noncancerous tissues. The PCA-LDA diagnostic algorithm has a sensitivity of 73.7% and a specificity of 72.2%. As PCA-LDA diagnostic algorithm used entire *ex vivo* range of reflectance spectra for classification by the algorithm, the PCA-LDA method gives a more comprehensive analysis for the dataset. Therefore, the diagnostic specificity of the PCA-LDA algorithm is slightly better than that of the *R*540/*R*576 method. So, the *R*540/*R*576 method is used as a kind of simple empirical methods. The discrimination results of PCA-LDA are more comprehensive and reliable. Therefore, reflectance spectroscopy could be one of the screening methods for nasopharyngeal carcinoma detection.

It is worth mentioning that *ex vivo* reflectance spectral intensities and absorption bands intensities of oxyhemoglobin in both normal and malignant tissues decreased over time [13]. Subhash et al. observed that there are some spectral differences between *ex vivo* and *in vivo* normal tissues [13]. To be a more effective diagnosis method, the features of *in vivo* nasopharyngeal carcinoma from that of noncancerous one need further investigation with noninvasive *in vivo* detection in the future.

5. Conclusions

In summary, we studied the characteristics of reflectance spectra from benign and malignant *ex vivo* tissues in the human nasopharynx for the first time. Mean reflectance spectra of benign and malignant *ex vivo* tissue showed significant differences in the absorption bands from oxyhemoglobin bands. Using PCA-LDA diagnostic algorithms, we got a sensitivity of 73.7% and a specificity of 72.2% for NPC detection, indicating the potential of reflectance spectroscopy for *ex vivo* diagnosis of nasopharyngeal malignant tumor. In order to differentiate NPC from noncancerous *ex vivo* tissue based on reflectance spectra simply, mean spectral intensity ratios of oxyhemoglobin (*R*540/*R*576) were employed as an indicator of cancerization associated transformation in the hemoglobin oxygenation. These results demonstrated that reflectance spectroscopy combined with multivariate statistical tools can be used to indicate the inherent information like blood hemoglobin content, which has the potential to detect nasopharyngeal cancerous tissues quickly in real time, presenting a significant experimental and clinical value for further nasopharyngeal cancer detection *in vivo* in the future.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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