Partial Least-Squares Discriminant Analysis on Autofluorescence Spectra of Oral Carcinogenesis

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A partial least-squares (PLS) discriminant analysis on the autofluorescence spectra of oral squamous cell carcinoma based on the cross-validation technique was conducted to discriminate among oral tissues at different cancer development stages. These tissues were obtained from hamsters of DMBA-induced buccal pouch carcinogenesis. The study on the fluorescence spectra of the cancer tissues revealed that 320 nm might be the optimal excitation wavelength, and it was selected for the discriminant analysis. The PLS discriminant plot based on cross-validation showed that tissues of oral carcinogenesis belonging to four clinically important cancer development stages—normal tissues, hyperplasia, dysplasia and early cancers, and frankly invasive cancers—could be classified by using the first two PLS factors that emerged from the fluorescence spectra at 320 nm excitation. The PLS factor loading plots of the first PLS factor of 320 and 360 nm excitations showed that the first PLS factor was correlated to the fluorescent structure changes. This study indicates that further development of the PLS discriminant analysis on the autofluorescence spectra may be useful for developing a simple and efficient discriminating algorithm for the identification of different stages of human oral carcinogenesis.

Index Headings: Autofluorescence spectroscopy; Hamster buccal pouch carcinogenesis; Animal model; Partial least-squares discriminant analysis; Oral squamous cell carcinoma.

INTRODUCTION

Autofluorescence spectroscopy, a new technique for differentiating cancerous tissues from normal tissues, has been studied by many researchers. \(^1\)\(^-\)\(^3\) Compared with conventional biopsy techniques, autofluorescence spectroscopy has many advantages such as minimum invasion, real-time diagnosis, and no need for adding fluorescent dyes. \(^4\) Furthermore, autofluorescence spectra can uniquely display the spectral features characterizing the state of cells that make up tissues. \(^5\) Therefore, autofluorescence spectra can be used to detect changes in cellular chemistry and tissue architectures associated with the development of cancers. \(^6\) On the basis of these reasons, autofluorescence spectroscopy has the potential to be an efficient tool for discriminating tissues among different stages of cancer development.

The patterns of autofluorescence spectra depend on the biochemical and biophysical structure of tissues. \(^1\) Many researchers have pointed out that tissue autofluorescence mainly comes from natural fluorophores such as tryptophan, tyrosin, elastin, collagen, NADH, FAD, and so on. \(^1,7,8\) Because the structures of these molecules are different, complex, and all present in the system, the autofluorescence spectra of tissues are usually broad-band in nature. This fact indicates that it will be more informative to use the full spectrum than to select only some points of the spectrum for analysis. For this purpose, multivariate statistical methods are often used for spectrum analysis. \(^9\) However, fluorescence spectra usually involve a large number of correlated variables, so a variable reduction technique is required to eliminate the colinearity in the spectral data and to simplify the data analysis.

In the present research, partial least-squares (PLS) discriminant analysis \(^10\) was adopted for spectrum analysis. PLS analysis and principal component analysis (PCA) are two commonly used multivariate statistical methods for spectral analysis. Similar to PCA, the PLS discriminant analysis creates new factors that account for most variance of the data set; thus the important information can be extracted by using far fewer variables. \(^3,11\) However, PLS discriminant analysis differs from PCA in that the former utilizes the known group association of samples during the factor extracting process. \(^12,13\) In general, PLS discriminant analysis can achieve better classification performance with fewer factors than PCA. \(^13\)

In this research, we studied the autofluorescence spectra of hamster buccal pouch tissues from a 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral carcinogenesis animal model. This hamster buccal pouch carcinogenesis model was first described by Sally in 1954, \(^14\) and was modified and standardized by Morris to make it possible to uniformly reproduce the lesions at a later time. \(^15\) It is a well-known and useful animal system that closely resembles events in the development of precancerous lesions and squamous cell carcinoma (SCC) in the human oral cavity. Recent work done by Balasubramanian et al. demonstrated that the DMBA-treated hamster buccal mucosa showed hyperplastic changes at 4–6 weeks, papillomas at 8–10 weeks, early invasive SCC at 11–13 weeks, and well-differentiated SCC at 14–16 weeks of treatment. \(^5\) Thus, the DMBA-induced hamster buccal pouch carcinogenesis is a good model for studying the autofluorescence spectra for progression of oral cancer.

Some researchers have shown that there are significant differences in the autofluorescence spectra between normal and cancerous human oral tissues. \(^16,18\) However, the identification of fluorescence spectra of tissues at various cancer development stages has not been well studied. In order to establish autofluorescence spectra data sets of different cancer development stages for analysis, a DMBA-induced hamster buccal pouch carcinogenesis model was used in the present study. An animal model

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can simplify the involved factors and provide tissues of an appropriate number at different oral carcinogenesis stages. In 1995, Balasubramanian et al. conducted a study on the fluorescence spectra by extracting some fluorophores from the hamster buccal pouches treated with DMBA and observed that the fluorescence spectra have significant differences among normal, premalignant, and malignant tissues when excited at 405 and 420 nm. Instead of extracting fluorophores from samples, we measured the fluorescence spectra of the whole excised tissues to simulate the in vivo measurement and further analyzed the full fluorescence spectra by using PLS discriminant analysis and tissue autofluorescence.

The goal of the present study was to discriminate oral tissues among different cancer development stages by using PLS discriminant analysis. To address this objective, we measured the autofluorescence spectra of the hamster buccal pouch tissues at different DMBA application periods at various excitation wavelengths. The optimal excitation wavelength for discriminating the fluorescence spectra among different cancer development stages was selected by calculating the minimum residuals of the PLS discriminant analysis based on cross-validation. Also, the PLS scores obtained on the basis of cross-validation at the optimal excitation were used for constructing a PLS discriminant plot, which shows that this technique is useful for the classification of samples at different cancer development stages.

**EXPERIMENTAL**

**Sample Processing.** Forty-eight four-week-old Syrian male hamsters were used for DMBA-induced carcinogenesis. These hamsters were painted with a 0.5% solution of carcinogen DMBA in heavy mineral oil on their left buccal pouches three times a week. The right buccal pouches of these hamsters were left untreated.

During the complete course of the DMBA application, six hamsters were sacrificed every two weeks. Both the left and right buccal pouches of these hamsters were excised, and the appropriate tissue samples were taken and divided into two pieces measuring from 4 to 6 mm in diameter and from 0.8 to 1.2 mm in thickness. One piece was embedded in cryomatrix (Shandon Scientific Ltd., England), stored in liquid nitrogen within 5 min after sacrifice, and later in an ultralow temperature freezer (−70 °C) until the time of fluorescence measurement. The other piece was fixed in 10% neutral formalin, embedded, sectioned, H&E stained, and sent for histological examination by two oral pathologists (Dr. C. P. Chiang and Dr. J. T. Wang). In addition, after autofluorescence measurements, all the tissue samples were treated by the above-mentioned routine histological procedures and sent to the same oral pathologists for confirmation of the diagnosis.

**Histology.** The histological assessment showed that hyperkeratosis and acanthosis with some focal mild epithelial dysplasia appeared in the samples after 2 to 6 weeks of DMBA application. Moderate or severe epithelial dysplasia appeared after 8 weeks of DMBA application. Carcinoma in situ together with some papillary or early invasive squamous cell carcinoma developed after 10 to 12 weeks of DMBA application. Various sizes of tumors, which were proved to be frankly invasive SCC histologically, were found on the left buccal pouches after 14 to 16 weeks of DMBA application. Similar results have also been observed by Balasubramanian et al.

There were no definite histological changes observed in the right buccal pouches of all the hamsters. Thus, the samples of right buccal pouches served as normal control specimens in this study.

**Sample Classification.** Table I shows the classification and number of the samples at different cancer development stages. Group I represents normal samples. Group II represents samples of hyperkeratosis, acanthosis, and mild epithelial dysplasia. Group III contains samples of moderate or severe epithelial dysplasia, carcinoma in situ, and early invasive carcinoma. Group IV consists of samples of papillary and frankly invasive SCCs. These four groups stand for important cancer development stages: normal tissues, hyperplasia, dysplasia and early cancers, and frankly invasive cancers.

**Fluorescence Measurement.** Before fluorescence measurements, the frozen samples were brought to room temperature and rinsed with 4 °C phosphate buffered saline (PBS, pH = 7.4) to remove the remaining embedding medium and to keep them moist. A standard luminescence spectrometer (SLM Instruments, Urbana, IL, U.S.A.) was used for fluorescence measurement. During the fluorescence measurement, the samples were mounted on the backside of a quartz slide, placed in the cuvette holder of the spectrometer, and excited by UV light at 37° incident angle. The fluorescence was collected at 90° normal to the excitation light. Such an arrangement can effectively reduce the collection of the excitation light reflected from the surface of the quartz slide or the sample. The incident beam was focused on the center of tissue surface with a spot size of 4 mm × 1 mm, and the incident power ranged from 3 μW (280 nm excitation) to 27 μW (400 nm excitation). In the autofluorescence emission scan measurement, the excitation light was changed from 280 to 400 nm in 20 nm increments. The emission spectra were measured from 20 nm above the excitation wavelength to 20 nm less than twice the excitation wavelength in 3 nm increments. The refresh period for data acquisition was 0.5 s, and the scan rate of emission spectra was set at 18 nm per second. Less than 10 min was required for the measurement of each sample. While measured, the samples were kept moist, and a repeated emission scan was conducted at 280 and 300 nm excitations to monitor tissue deterioration and photo-bleaching. Less than 1% change in the intensities of the re-scanned spectra was observed. All the fluorescence spectra were normalized by dividing the intensity at each

<table>
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<tr>
<th>Classification</th>
<th>Cancer development stages</th>
<th>Sample number</th>
</tr>
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<tbody>
<tr>
<td>Group I</td>
<td>Normal</td>
<td>48</td>
</tr>
<tr>
<td>Group II</td>
<td>Hyperkeratosis, acanthosis, and mild epithelial dysplasia</td>
<td>18</td>
</tr>
<tr>
<td>Group III</td>
<td>Severe epithelial dysplasia, carcinoma in situ, and early invasive squamous cell carcinomas</td>
<td>18</td>
</tr>
<tr>
<td>Group IV</td>
<td>Papillary and frankly invasive squamous cell carcinomas</td>
<td>12</td>
</tr>
</tbody>
</table>
wavelength by the integrated area under the total spectrum.

RESULTS AND DISCUSSION

Properties of the Fluorescence Spectra. Figure 1 depicts the normalized and averaged spectra of the samples with the same durations of DMBA application. For simplification, only the spectra of normal samples and 4, 8, 12, and 16 weeks of DMBA-treated samples at 280, 320, and 360 nm excitations are shown in the figure. It is worth noting that different excitations resulted in different patterns of fluorescence spectra. Figure 1a shows a prominent emission peak near 330 nm at 280 nm excitation, but few differences are found among these spectra. Figure 1b shows two emission peaks near 385 nm and 470 nm at 320 nm excitation. The first peak (near 385 nm) decreased while the second peak (near 470 nm) increased with longer DMBA application duration. This change might be due to collagen decrease and NADH increase in cancer tissues. Figure 1c shows two prominent emission peaks near 460 and 640 nm at 360 nm excitation for the samples with 10 to 16 weeks of DMBA treatment. The first peak (near 460 nm) decreased while the second peak (near 640 nm) increased with longer DMBA application duration. The collagen decrease and porphyrin compound accumulation might be related to the changes in these fluorescence patterns. The autofluorescence spectra of the samples treated by different durations of DMBA exhibited significant differences at 320 nm and 360 nm excitation, but only minor differences were noted at 280 nm excitation.

Understanding the fluorescent structure in normal and cancer tissues is important for cancer diagnosis by using autofluorescence spectroscopy. Some researchers declared that the autofluorescence changes in cancer tissues might be mainly due to tissue architectural differences (e.g., the decrease in collagen or increase in NADH); while others suggested that the alternation of autofluorescence for cancer tissues might be caused by specific fluorophores in tissues (e.g., the porphrine compounds accumulation). A recent work using laser-induced fluorescence microscopy on colon tissues shows that there are intrinsic differences in tissue autofluorescence that are attributable to both tissue architecture changes and specific fluorophores. In the future, using microspectrofluorometric analysis on tissue sections may be helpful for improving the accuracy of the autofluorescence spectroscopic diagnosis technique for cancer.

In this study, all tissues were frozen 5 min immediately after the hamsters were sacrificed. A preliminary experiment that was conducted to monitor the autofluorescence alterations between the fresh and frozen samples showed less than 3% change in the intensities between two kinds of samples. According to Schomaker et al. the fluorescence intensity of NADH decays with a time constant of 118 min after resection. Since the tissues were frozen within 5 min after sacrifice, the time that elapsed between the resection and the freezing of samples might not affect the data significantly.

PLS Discriminant Analysis. The algorithm of PLS discriminant analysis is based on the projection of multivariate data sets into far fewer dimensions that are most relevant to the known group association of samples. PLS discriminant analysis has been widely used for multivariate analysis in many fields. Figure 2 is a schematic diagram describing the PLS algorithm. In the figure, \( X_m \) and \( Y_m \) are the mean-centerized matrices of data matrix \( X \) and dependent variable matrix \( Y \), respectively. Matrices \( E \) and \( F \) are the residuals after the PLS factors are extracted. Matrices \( T \) and \( U \) are the PLS scores. First, pairs of PLS factors are extracted from \( X_m \) and \( Y_m \), and the additional pairs of PLS factors can then be calculated from residuals \( E \) and \( F \) sequentially. Thus the PLS factors are determined to describe \( X_m \) as well as possible and simultaneously to correlate with \( Y_m \).

The samples were divided into 12 subsets; each subset has nearly the same number of samples belonging to four cancer development stages (Table I), and the validation process was repeated 12 times. The data matrix \( X \) is created with the spectra of samples in the calibration set for
one excitation wavelength. Each row of the matrix contains the autofluorescence spectrum of a sample, and each column contains the autofluorescence intensity at each emission wavelength. The four cancer development stages were coded as three dummy variables (Table II) to construct a dummy matrix $Y$. Let the $i$th PLS scores for all of the 96 samples be placed in a vector $t_i$, and $\|t_i\|$ represent the Euclidean norm of $t_i$. Thus, larger $\|t_i\|^2$ indicates more information contained in $t_i$.

Let's define $v_i$ in the following equation:

$$v_i = 100 \times \left( \frac{\|t_i\|^2}{\sum \|t_i\|^2} \right).$$

(1)

Thus those $t_i$'s having larger $v_i$ account for more information, and those PLS scores in $t_i$ are more appropriate for generating the PLS discriminant plot.

Figure 3 shows the $v_i$'s for the first four PLS factors at various excitation wavelengths. The values of $\|t_i\|^2$ ($i > 25$) were at least $10^6$ times smaller than $\|t_i\|^2$; thus the summation was calculated up to the 25th PLS factor. In the figure, we see that the first four PLS factors together account for most of $\sum_i \|t_i\|^2$ (more than 97.5%; i.e., $\sum_{i=1}^4 v_i > 97.5\%$) for all the excitation wavelengths. All $t_i$ ($i > 4$) accounted for a very small portion of $\sum_i \|t_i\|^2$ (less than 1%); thus they were not considered to be significant and are not shown in Fig. 3.

Figure 4 shows the relationship between the root mean square error of cross-validation (RMSECV) and the number of PLS factors for different excitation wavelengths. The RMSECV is defined to be the average of the root mean square error of cross-validation for individual dummy variable:

$$\text{RMSECV} = \frac{1}{3} \sum \text{RMSECV}_i = \frac{1}{3} \sum \sqrt{\frac{\sum_i (y_{i,t} - y_{i,k})^2}{N}}$$

(2)

where $N$ is the number of samples; $y_{i,t}$ represents the predicted value of $y_{i,k}$, which stands for the expected output of the $i$th sample for the $k$th dummy variable. Since each $t_i$ ($i > 4$) was not considered to be significant, only the RMSECVs of the first four PLS factors are shown in Fig. 4.

**TABLE II.** The coded variables settings for the four groups.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Dummy variables in dummy matrix $Y$</th>
</tr>
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<tbody>
<tr>
<td>Group I</td>
<td>0 0 0</td>
</tr>
<tr>
<td>Group II</td>
<td>0 0 1</td>
</tr>
<tr>
<td>Group III</td>
<td>0 1 0</td>
</tr>
<tr>
<td>Group IV</td>
<td>1 0 0</td>
</tr>
</tbody>
</table>

![Graph showing the relationship between the number of PLS factors and the root mean square error of cross-validation at various excitations.](image-url)
4. We found that the RMSECVs of 320 nm excitation are always the smallest with the use of either two, three, or four PLS factors. Thus we conclude that 320 nm might be the optimal excitation wavelength for classifying the samples. Therefore, the PLS factors extracted from the fluorescence spectra at 320 nm excitation were chosen to construct the PLS discriminant plot and the PLS factor loading plot.

**PLS Discriminant Plot.** Figure 5 shows the bar chart of $|t_i|^2$ for 320 nm excitation. The results were obtained from the PLS scores based on cross-validation. Because $|t_1|^2$ and $|t_2|^2$ are much larger than other $t_i$'s, the first two PLS factor scores were chosen for constructing the PLS discriminant plot. Figure 6 shows the PLS discriminant plot of the first two PLS factor scores ($t_1$, $t_2$) obtained from the fluorescence spectra based on cross-validation at 320 nm excitation. The four groups are encircled so that the group structures can be visually observed. The 96 samples could be well classified into four groups by means of $t_1$ and $t_2$ but the class structures of these groups showed great differences. It is worth noting that the variations of samples become larger and larger from group I to group IV. This fact indicates that the variations of fluorescence spectra are not very significant among normal tissues but gradually become apparent during the cancer development. Because the changes in the fluorescence spectra of the cancer tissues involve complex biochemical and biophysical mechanisms, the patterns of the fluorescence spectra have great diversity.

In Fig. 6 the classification rates of samples belonging to group III are relatively lower than those of samples belonging to groups I, II, and IV. This observation may be due to the diverse nature of samples in group III, which contain tissues varying from severe epithelial dysplasia to early invasive SCC. The unsatisfactory results in group III might be due to the collection of a variety of different samples with a small sample population. The variation from severe dysplasia to early invasive carcinoma of the 18 samples in group III might cause the variations of the PLS scores of these samples.

Regarding the problem in this *ex vivo* study, we plan to do a clinical *in vivo* study, in which a large number of data sets can be obtained. We may classify sufficient samples into more groups representing various transitional stages; thus the PLS discriminant analysis can be improved.

**PLS Factor Loading Plot.** Figures 7a and 7b show the PLS factor loading plot of the first PLS factor obtained from the fluorescence spectra at 320 and 360 nm excitations. The PLS factor loadings describe the correlation between the PLS factors and the original variables (the emission wavelengths in this study). The emission wavelengths with a large absolute value of the factor loading are more important in establishing the statistical model. Because of no significant differences, the data were obtained from one of the calibration sets based on...
Fig. 7. The PLS factor loadings plots for (a) 320 nm and (b) 360 nm excitations. The wavelengths of the peak values are also shown in the figures. The results are from one of the calibration sets of cross-validation.

the cross-validation. In this figure, we found that peaks appeared at about 390 and 470 nm at 320 nm excitation, and at about 460 nm and 640 nm at 360 nm excitation. These results indicate that the first PLS factor is correlated to the fluorescent structure changes, including the decrease in collagen, increase in NADH, and porphyrin compounds accumulation.

**Applicability on Human Oral Cancer.** The hamster buccal pouch carcinogenesis model is a well-known system for the study of development and therapy of human oral carcinoma. In particular, the DMBA-induced hamster buccal pouch carcinogenesis, the consecutive order of hyperkeratosis and acanthosis, dysplasia, early invasive carcinoma, and frankly invasive SCC, has been shown to closely resemble the development of SCC in the human oral cavity. Furthermore, among the types of human oral cancer, SCC is the most widespread one. Thus, the results in this study, including the progression of oral cancer, the classification of cancer stages, and the changes of autofluorescence spectra for cancerous tissues, might be useful for human oral cancer identification.

The technique and algorithm developed in this study might be better than or complementary to some existing cancer diagnostic techniques such as biopsies and subsequent pathological examination. For example, this technique could be developed to clinically determine whether there are residual tumor tissues at the section margins after removal of the main tumor mass, or to detect residual cancer tissues when the lesions disappear after chemotherapy or radiotherapy. In addition, with the combination of autofluorescence spectroscopy and the toluidine blue vital stain technique, the accuracy of noninvasive cancer detection might be much improved.

Unlike the specular method, autofluorescence spectroscopy can be algorithmized to differentiate normal from cancerous tissues automatically. A user-friendly system might be useful for routine clinical screening of oral cancer by integrating autofluorescence spectroscopy and the appropriate diagnosis algorithm. It might be especially helpful for less experienced practitioners, because this technique could provide quantitative and objective data and might have the potential to help doctors determine the suitable sites of lesions for biopsy. Therefore, this approach could not only save patients from multiple biopsies but also allow more wide-scale diagnosis.

**CONCLUSION**

In this study, a total of 96 buccal pouches of hamsters were assigned to four groups based on their histological assessment results, and their fluorescence spectra were used for PLS discriminant analysis. By using the PLS discriminant analysis based on cross-validation, we found that the autofluorescence spectra at 320 nm excitation might be optimal for classifying the samples into different cancer development stages. A PLS discriminant plot using the first two PLS factors scores ($t_1$, $t_2$) could be employed to classify the samples into four groups, which represent four clinically important cancer development stages: normal tissues, hyperplasia, dysplasia and early cancers, and frankly invasive cancers. However, the sensitivity for identifying dysplasia and early cancer is relatively low, indicating that the sample population may need to be enlarged. This result indicates that a further development of PLS discriminant analysis based on autofluorescence spectra may be useful for the identification of different stages of human oral carcinoma.

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8. N. Ramanujam, M. F. Mitchell, A. Mahadevan, S. Warren, S.