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COMMUNICATION

Application of Linear Discriminant Analysis and Attenuated Total Reflectance Fourier Transform Infrared Microspectroscopy for Diagnosis of Colon Cancer

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Abstract Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) microspectroscopy was applied for detection of colon cancer according to the spectral features of colon tissues. Supervised classification models can be trained to identify the tissue type based on the spectroscopic fingerprint. A total of 78 colon tissues were used in spectroscopy studies. Major spectral differences were observed in 1,740–900 cm^{-1} spectral region. Several chemometric methods such as analysis of variance (ANOVA), cluster analysis (CA) and linear discriminate analysis (LDA) were applied for classification of IR spectra. Utilizing the chemometric techniques, clear and reproducible differences were observed between the spectra of normal and cancer cases, suggesting that infrared microspectroscopy in conjunction with spectral data processing would be useful for diagnostic classification. Using LDA technique, the spectra were classified into cancer and normal tissue classes with an accuracy of 95.8%. The sensitivity and specificity was 100 and 93.1%, respectively.

Keywords Colon cancer · Diagnosis · ATR-FTIR · Chemometrics · LDA

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Introduction

Cancer is one of the main causes of death all around the world and it will soon be the leading cause of death. In the United States, over 1.5 million cases of cancer are estimated to be diagnosed in 2009 [1]. Colon cancer has allocated one of the highest mortality rates. Abnormal cell proliferation has been known to be an indicator of the initiation of malignancy [2]. The detection of abnormal crypts becomes essential for diagnosis of colon cancer and its effective management [3, 4]. Hitherto the detection of premalignant conditions in the colon has been associated with abnormal crypt proliferation [5–8]. Various techniques have been utilized to identify abnormal crypt proliferation in colon tissues [9, 10]. The presence of certain molecular markers in the crypt has been used to detect abnormal proliferation [7, 8, 10, 11]. In some studies, abnormality was identified using sensitive markers, which were defined at molecular levels [12]. Despite the identification of certain specific markers for abnormal cell proliferation [13], the actual maturation of the crypt in terms of total biochemical changes has not been established. The "gold standard" in most cancer diagnostics is microscopic evaluation, by a pathologist, of a stained tissue obtained from biopsy of a particular organ. The differences between "malignant" and "normal" have many observations in common e.g. morphology and size of cells that are different and more variable than those of normal cells, nucleus of a cell that is larger than the nucleus of a normal cell, large cells with multiple nuclei, and the invasion of normal tissue by a neoplasm. Although many pathologists are exceptionally good at what they do, this analysis is somewhat subjective. "Misdiagnosis", with a false negative or false positive result is common in tissue evaluation. Additionally, in some cases (about 10%), a pathologic examination may not produce a

firm diagnosis, either because certain tumors are histologically similar or because cells are so poorly differentiated that their tissue of origin cannot be determined. In these cases, other diagnostic procedures might be useful and include, but are not limited to, electron microscopy, immunohistochemistry, cytogenetics, and levels of various tumor markers in the patient's serum or urine, and nowadays infrared spectroscopy [14, 15].

One of the main advantages of infrared spectroscopy is that it is not limited to a particular state of the sample. Spectra can be obtained from liquids, solids (pellets, powders, films and tissues), slurries, and suspensions. IR spectroscopy is well known for its uniqueness as a nondestructive method in identifying vibrational structure of various materials. The spectra allow measuring complex molecular vibrational modes. Various biomolecular components of the cell give a characteristic IR spectrum, which is rich in structural and functional aspects [16, 17]. One of the most promising applications of the IR-based techniques, which have become possible now, is in biomedicine. IR spectroscopy can detect and monitor characteristic changes in molecular composition and structure that accompany transformation from normal to cancerous state. IR spectroscopy opens new and modern areas of medical research, as it causes no damage to the cells [17–22].

The identification of an absorbance intensity peak or ratio of peaks may allow the identification of a biomarker for pre-malignancy or cancer. Of course it is of high importance to mention that the quantity of the desired biomarker must be in the detection limit of IR spectrometric techniques. Attenuated total reflection-Fourier transform infrared microspectroscopy allows the fast acquisition of spectra with a high signal to noise ratio (SNR) and allows the interrogation of individual groups of cells, high-density samples, and complex cell membranes [23].

The results of different researches support the idea that major biochemical changes are taking place in the cells undergoing transformation from normal to cancerous state. The FTIR microspectroscopy is capable of detecting malignancy among colon cancer patients using biopsy tissue samples. There are some other useful techniques e.g. MALDI-MS, and NMR which are also suitable for diagnostic approaches with higher selectivity and sensitivity according to their imaging and mapping capabilities. FTIR spectra of normal and malignant cells from tissue samples of different colon cancer patients show the presence of two families: normal and abnormal cells. The splitting pattern has been observed in 1,150–1,000 cm^{-1} spectral region for normal cells and are absent in polyp and malignant cells. However, an extensive set of spectroscopic changes are common between the malignant tissue and colon adenocarcinoma cell lines. Studying the colon cancer cell lines demonstrate several of the spectroscopic features displayed by colon cancer tissues [24]. Cell lines have been as an appropriate experimental system both to study the origin of the spectroscopic changes observed hitherto and to apply the IR technology to other aspects of cancer biology. Malignant colon tissues can be also distinguished from normal colon tissues by a series of high-pressure infrared spectroscopic parameters. Some infrared parameters of malignant colonic tissue obtained at atmospheric pressure are significantly different from those of normal colonic tissue e.g. a 2–3 cm^{-1} difference in the frequency of the symmetric phosphate stretching band of the nucleic acids between normal and malignant colon tissues [25, 26].

Infrared spectrum database of colorectal cancer patients has been investigated as a diagnostic approach [27]. IR imaging in mid and near region in addition with data processing is also a reported idea for colon cancer analysis which has been compared with raman results. NIR fluorescence is capable of deep tissue analysis and could be proposed a more sensitive technique [28–32]. Literature survey shows that most of the research performed in the field of cancer detection by FTIR spectroscopy is based on experimental investigation of isolated signal intensities or signal position shift and related signals' intensity ratio. It is noticeable that nowadays many chemometric techniques are applied while research is performed in order to combine the statistical skills with chemical ones and gain as insurable as possible results. Multivariate data analysis is one of these techniques that has been presented by several researchers [33]. "Chemometrics" is a chemical discipline that uses mathematical and statistical methods to design or select optimal measurement procedures and experiments, and to provide maximum chemical information by analyzing chemical data. One of the first and the most publicized success stories in chemometrics is pattern recognition. Much chemistry involves using data to determine patterns. There are a large number of methods for supervised pattern recognition, mostly aimed at classification. Multivariate statisticians have developed many discriminant functions, some of direct relevance to chemists. In considering a chemical method such as FTIR spectroscopy to determine whether a sample tissue is cancerous or not, a method can be set up in which the spectra of two groups, cancerous and non-cancerous samples, are recorded. Then some form of mathematical model is set up. As a standard model, finally by using the proposed model, the diagnosis of an unknown sample can be predicted.

Analysis of variance (ANOVA) is a collection of statistical models and their associated procedures, in which the observed variance is partitioned into components due to different explanatory variables. The purpose of ANOVA is to find out whether data from several groups have a common mean. That is, to determine whether the groups are actually different in the measured characteristic [34, 35].

The advantages of using multivariate methods in evaluation of environmental and biological data with respect to absorbance

region of the objects studied have been demonstrated. Cluster analysis [36] is the name given to a set of techniques that seek to determine the structural characteristics of a data set by dividing the data into groups, clusters, or hierarchies. Samples within the same group are more similar to each other than samples in different groups. Cluster analysis is an exploratory data analysis procedure [37, 38].

Supervised multivariate methods, such as linear discriminant analysis (LDA) are powerful tools to build rules of discrimination that are used later to identify new samples. LDA searches for the variables containing the greatest interclass variance and the smallest intraclass variance, and constructs a linear combination of the variables to discriminate between the classes. The rule is constructed with training set of samples, and further tested with the test set. In this research, we tried to study colon tissues ATR-FTIR microspectroscopy, proposing a treatment of the results by LDA, in order to improve the reliability of the interpretation of the data [33, 35]. The linear combination for a discriminant analysis is derived from an equation as:

$$Z = w_1X_1 + w_2X_2 + w_3X_3 + \cdots + w_nX_n$$

where Z is the discriminant score, w_i is the discriminant weight for independent variable i and X_i is the independent variable i; in this case the independent variable corresponded to the absorbance in a wavenumber. Each w_i was set so as to maximize the between-group variance of Z (Z variance between cancer group and normal group) relative to the within-group variance of Z (Z variance within cancer group and the variance within normal group). The critical Z-value (z_{ce}) was then defined as:

$$z_{ce} = \ln\frac{n - n_{cancer}}{n_{cancer}} + \frac{1}{n}\sum_{j}^{n} z_j$$

where n cancer is the number in cancer samples, n is the number in all samples and zj is Z of the sample j. Based on $z$ce, cancer was segregated from normal tissue as follows:

$zj \geq z$ce the sample j is classified as 'cancer'
$zj < z$ce the sample j is classified as 'normal'.

The innovative aspect is based in the application of this chemometric tool, which provides a powerful device to extract useful information from the experimental data.

Methods and Materials

Colon Tissue Samples

A total of 78 formalin-fixed, paraffin-embedded colonic tissue specimens were obtained by the pathology division in Shahid Rajaee Hospital, Qazvin, Iran (associated with Qazvin University of Medical Science). Tissue section slides had been H&E stained and the paraffin removing procedure had been performed using xylene as the solvent. Patients were in 59–91 range of age, 42 males (average age: 78) and 36 females (average age 76) who underwent all pathologic test procedures. All the samples were obtained according to the proper ethical guidelines with permission obtained from all subjects. Patients were informed about the research goals and participated in the sampling voluntarily. It is noticeable that total number of samples was selected according to the cases which were in clinical relation with the hospital during the investigation time period. All the samples were investigated diagnostically by the pathology department of QUMS and the evaluation was repeated by second and third look. Thus the reliable resulted samples were chosen to be analyzed by FTIR spectrometry, while the pathology gold standard method's results were supposed as the reference. In order to obtain more precise data from cancerous tissues during the infrared microspectroscopic analysis and avoiding the probable misdiagnosis due to presence of wide range of pathological cell structures, the specified areas of tissue samples, confirmed by pathologic observations were located as the area for which the spectrum must be recorded.

FTIR Spectral Studies

All FTIR spectra were collected using an ABB-BOMEM spectrometer (Québec, Canada) equipped with a microscope (Spectratech®; Colorado, USA) at mid-IR region and ZnSe ATR accessory. The aperture size was $100 \times 100\ \mu m^2$ and a liquid nitrogen cooled mercury cadmium telluride (MCT) detector was used. In order to achieve the ideal signal to noise ratio (SNR), FTIR spectra were obtained by collecting 49 scans at a resolution of 8 cm^{-1}. An image of a normal and a cancer case sample is shown in Fig. 1.

Multivariate Statistical Analysis

In order to evaluate the results of ATR-FTIR microspectroscopy, the microspectroscopic study was paralleled with pathologic diagnostic study according to method of hematoxylin/eosin (H&E) staining. Then, obtained spectra were statistically analyzed to determine differences and convert the spectroscopic data into clinically useful information. LDA is a classification technique that employs Mahalanobis distance to determine the class of an unknown sample. In this study, training and test sets were selected randomly from the database. The training model was formed by 30 samples. Then 48 tissue samples were employed as the independent test. LDA was performed by TQ-Analyst® software (ThermoScientific®; Waltham, USA) and all other pre-processing methods were accom-

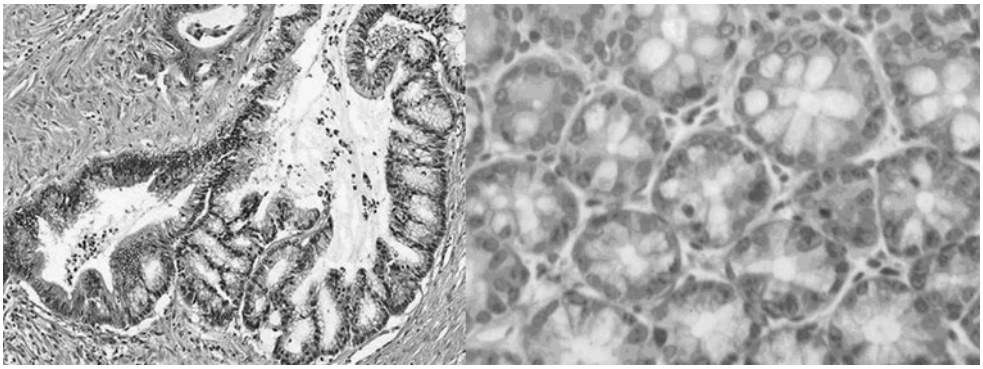

Fig. 1 Microscopic image of cancer (left–10x) and normal (right–20x) H&E stained colon tissues

plished using Matlab Version 7.0 (The MathWorks Inc., MA, USA).

Results and Discussion

Comparing the FTIR Spectral Features on Normal and Malignant Tissues

Cancerous colonic tissues show a systematic decrease in total carbohydrate, phosphate and possibly creatine content of cells [24]. Phosphate stretching modes could be useful as infrared spectroscopic markers to discriminate between spectra of normal and cancerous colonic tissues. Also, it is important to identify the special patterns of vibrational modes characteristic to various states of malignancy. Investigating typical infrared absorption spectrum of normal and cancerous human colon tissues demonstrates the main differences in 1,800–900 cm^{-1} spectral region. Typical spectra of normal and cancerous tissues are shown in Fig. 2. Although all biomolecules are important, the nucleic acids RNA and DNA are especially important because they carry within their structure the hereditary information that determines the identity and structure of proteins. The most significant signals for these nucleic acids in mid-IR region are in 1,800–900 cm^{-1}. The signal at 1,750–1,620 cm^{-1} corresponds to in-plane double-bond

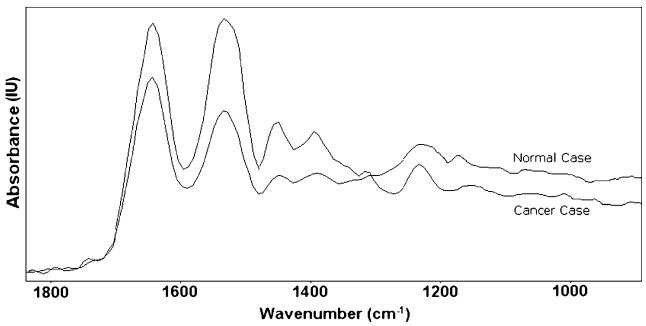

Fig. 2 Typical ATR-FTIR spectra of normal and cancer colon tissue in 1,800–900 cm^{-1} region demonstrating the spectral differences

vibrations of the bases. It is noticeable that the spectra in this region are very sensitive to base-pairing interactions and base-stacking effects, i.e. effects of hydrogen bond formation. As detailed in many other researches, the most significant signals are at 1,650 and 1,540 cm^{-1}, known as amide I and II, respectively [25, 27, 29, 30]. The absorption due to normal tissue was higher than cancerous types in this region of the spectrum in all patients. Amide I is due to the C=O hydrogen bonded stretching vibrations, and amide II is from the C–N stretching and a CNH bending vibrations. The intensity differences are the main object to be studied. Absorption bands at 1,240 and 1,080 cm^{-1} are in relation with the PO^{2-} ionized asymmetric and symmetric stretching respectively [23]. Other signals in this region are due to amino acid side chain from peptides and proteins at 1,460 and 1,400 cm^{-1}, associated with the asymmetric and symmetric CH_3 bending vibrations. Ribose has a strong C–O band at 1,120 cm^{-1}, which serves as a marker band for RNA. The bands at 1,025 and 1,045 cm^{-1} are responsible for the vibrational modes of $–CH_2OH$ groups and the C–O stretching coupled C–O bending of the C–OH groups. On the other hand, lipids, polysaccharides, and other biochemicals would also have many signals in the 1,800–900 cm^{-1} spectral region [39–42]. Thus, it is so difficult to evaluate the effect of single signals in the aforementioned spectral region as the markers for malignant sample detection. According to the complex structure of the spectra, it is important to consider the total region for better diagnosis. A key aim of experimentation is to ask how significant a factor is in colon cancer spectra. The use of ANOVA is widespread and is based on these simple ideas. Normally two mean errors are compared. It has been proved to be useful to apply chemometric techniques for better discrimination of different classes in chemical researches. ANOVA attempts to use a comparison of the estimates of variance from different sources data. These sources are the spectral data obtained by FTIR spectrometry to recognize whether the desired classes have a significant difference. If the data tends to demonstrate non-useful homogeneity, it is common to stabilize the model variance by data transformation. Figure 3 shows box plot achieved from ANOVA of two

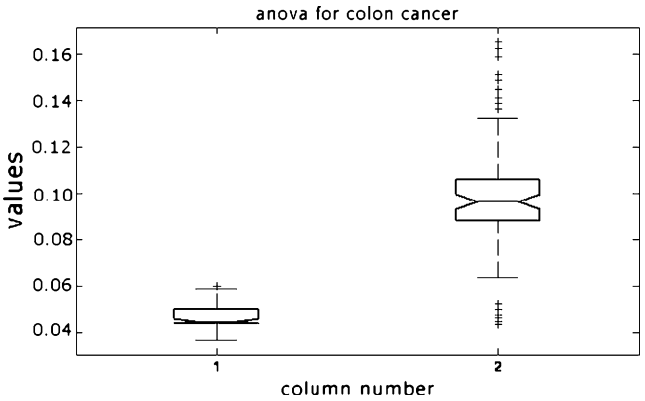

Fig. 3 Box plot for two classes of spectra (normal case in column 1 and colon cancer in column 2) demonstrating the obtained variance values and their distribution which confirms the capability of initial data to be classified

classes of cancer tissue spectra that illustrates use of infrared spectra in diagnosis of cancer is applicable. The difference between the obtained values is meaningful and confirms the capabilities for classification aims.

Cluster Analysis (CA)

Cluster analysis is a popular technique in which its basic objective is to discover sample groupings within data. The technique is encountered in many fields, such as biology, geology, and geochemistry, under such names as unsupervised pattern recognition and numerical taxonomy. The focus here is on hierarchical methods, as they are the most popular. For cluster analysis, each sample is treated as a point in an *n*-dimensional measurement space. The coordinate axes of this space are defined by the measurements used to characterize the samples. Cluster analysis assesses the similarity between samples by measuring the distances between the points in the measurement space. Samples that are similar will lie close to one another, whereas dissimilar samples are distant from each other. The choice of the distance metric to express similarity between samples in a data set depends on the type of measurement variables used. Measurement variables are usually continuous. For continuous variables, the Euclidean distance is the best choice for the distance metric, because interposing distances between the samples can be computed directly. Figure 4 shows dendrogram archived by application of CA into data set that indicates this method cannot separate two groups obviously.

Classification by LDA

The obtained FTIR spectra were statistically analyzed to determine differences between malignant status and normal status. LDA was applied to discriminate the two classes (normal and cancer). In order to reduce the number of variables in an infrared spectrum principal component analysis (PCA), unsupervised method was also performed. PCA optimized the number of PCs for LDA modeling. PCA removes the redundancy in the original data set such that the first few principal components (PC) scores are required to describe most of the information contained in all the original data Randomly, 30 tissue spectra consisting of 15 normal and 15 cancer cases were used as the training set for LDA modeling. The optimum model was tested by 48 other tissue spectra (29 normal and 19 cancer), which are not included in the training set. These 48 spectra were from 48 different samples and also 48 different patients. This

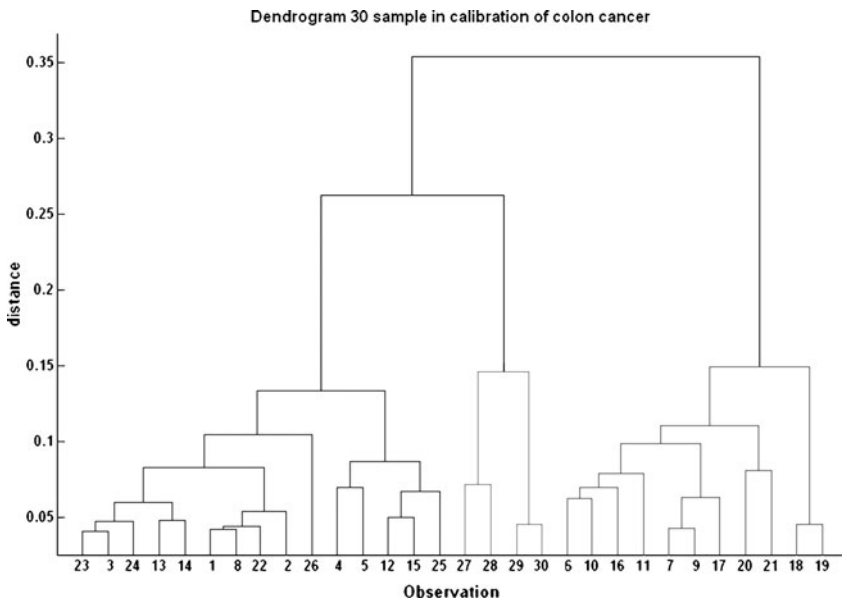

Fig. 4 The dendrogram of cluster analysis (with no clear classification) for 30 tissue samples (1–15 from cancer case and 16–30 from normal case) which was not useful for classification aim

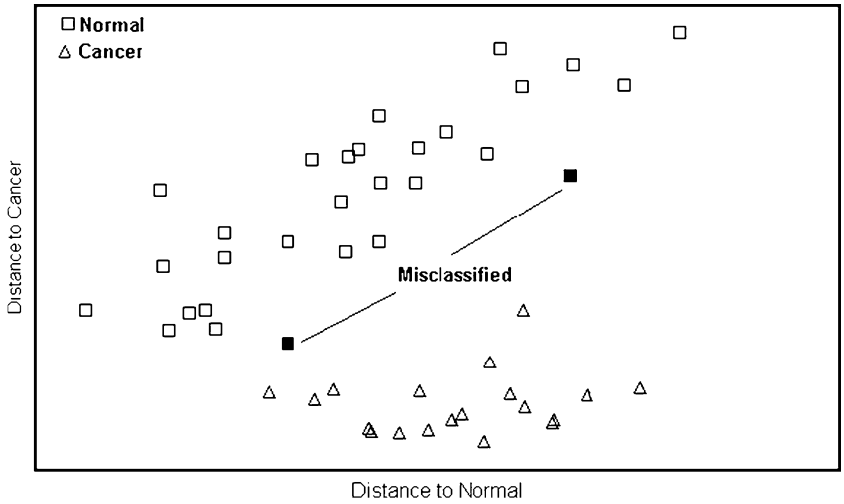

Fig. 5 Schematic classification of unknown colon tissues by LDA technique (misclassified samples have been highlighted)

strategy was decided in order to provide a more generalize able model. It is also noticeable that for each sample at least 3 spectra were recorded in order to monitor the repeatability. Results showed the prediction of 46 samples to be correct when compared with pathologic reports, while 2 normal tissue spectra were wrongly predicted to be cancerous and thus the accuracy was 95.8%. This rate of misclassification could be reasonable according to the limited database, in the other hand it could be due to the preliminary stage of the disease in the patients. It seems that a more populated model could solve this problem.

Statistical Analysis of FTIR Spectra

The spectral region of 1,740–900 cm^{-1}, a region in which many differences between cancer and normal tissues have been demonstrated in previous studies [17, 20, 21, 26, 27] was selected for the analysis. Discriminant analysis involves deriving a variant, the linear combination of the two (or more) independent variables that will discriminate best between a priori defined groups.

Figure 5 shows the schematic result of applying LDA technique for tissue spectra classification. It is observed that two normal samples have been predicted as cancer case. There are several probable reasons for misdiagnosis such as: undersized analyzed area during FTIR microspectroscopy studies which complicates the spectrum of cancerous area with healthy areas; limited difference between the cancerous and normal cells due to initial stages of cancer and also lack of repeatability in spectrum acquisition. The results obtained on data reported in this study support the idea that as major biochemical changes are taking place in the cells undergoing transformation from normal to cancerous state, it is more appropriate to have a total region analysis. Cancer case colon tissues show a systematic decrease in several biochemicals e.g. proteins and carbohydrates. LDA technique helps evaluating FTIR microscopy as a fast, accurate and easy to perform method detection of colon cancer and to get a better insight on the differences between normal and cancerous tissues. According to misclassification of two normal samples in cancer class (in totally 48 tissue spectra consisting of 29 normal and 19 cancer cases), sensitivity and specificity of the proposed method were determined to be 100 and 93.1%, respectively.

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