

FT-IR MICROSCOPIC CHARACTERIZATION OF NORMAL AND MALIGNANT HUMAN COLONIC TISSUES

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Abstract - Fourier-transform infrared spectroscopy (FT-IR) employs a unique approach to optical diagnosis of tissue pathology based on the characteristic molecular vibrational spectra of the tissue. In this study, we report infrared absorption spectra of formalin fixed, paraffin embedded normal and malignant human colonic tissues from ten different patients. Our method is based on microscopic infrared study (FT-IR-microscopy) of thin tissue specimens in parallel with normal histopathological analysis, which serves as a reference. Our results indicate that the normal colonic tissue has a stronger absorption than the cancerous type over a wide region in all ten cases. The detailed analysis showed that there is a significant decrease in carbohydrate levels, total phosphate and also possibly creatine contents for cancerous tissue in comparison to the controls. Also, RNA/DNA ratio increased in cancerous tissues relative to the normals in all the patients. The results of Linear Discriminant Analysis (LDA) showed that the normal and malignant cells could be identified with about 89% accuracy.

Key words: FT-IR microspectrometry, colon cancer, histology review, band fitting

INTRODUCTION

Of the estimated 5.2 million deaths from cancer per year in the world, 55% (2.8 million) occurs in developing countries. Lung cancer is still the most common cause of death from cancer worldwide with over 900,000 deaths per year, followed by gastric cancer with over 600,000 deaths and colorectal and liver cancers accounting for at least 400,000 deaths each (25). The ACS (American Cancer Society) estimates that there will be 93,800 new diagnoses of colon cancer and 36,400 new diagnoses of rectal cancer in the year 2000 in the USA (26). Despite the improvement in diagnostic techniques, the vast majority of pancreatic cancers, more than 90%, have either advanced or metastasized by the time they are diagnosed (1). Hence there is an urgent need to develop novel diagnostic method to detect the malignancy in the earlier stage.

Infrared (IR) spectroscopy is well known for its uniqueness as a non-invasive method in identifying vibrational structure of various materials (2). The spectra allow the study and identification of vibrational modes. Various biomolecular components of the cell give a characteristic IR spectrum, which is rich in structural and functional aspects (16,22). One of the most promising applications of the IR-based techniques, which has 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 (3,9,13). Literature has numerous examples including lung (8) cervix (30), prostate (21) and breast (4,5,12) and this growing list illustrates the potential of FT-IR in the field of infrared cancer diagnostics (optical biopsy).

There are only very few reports on diagnosis of colorectal cancer by Rigas and others using conventional FT-IR and microscopic FT-IR, respectively (17,27). FT-IR microscopic imaging of human colon carcinoma has also been reported (19). In this report, we describe the results on microscopic FT-IR of normal and malignant tissues of

Abbreviations: CCD: charged coupled detector; DNA: deoxy ribonucleic acid; FT-IR: Fourier transform infrared; MCT: mercury cadmium telluride; RNA: ribonucleic acid

human intestine from ten patients. Significant spectral differences have been observed between normal and cancerous tissues. The detailed analysis showed that phosphate content and RNA/DNA ratio may be potential markers to differentiate normal from diseased tissues. Linear discriminant analysis (LDA), used in the present study, provided good classification between normal and malignant cells. Also, the possible reasons for intensity variations between normal and cancerous tissues have been discussed.

MATERIALS AND METHODS

Sample preparation

Formalin-fixed, paraffin-embedded tissue from 10 adenocarcinoma patients, were retrieved from the histopathology files of Soroka University Medical Center, Beer Sheva (SUMC). The tissue samples used in this study were selected to include both normal and malignant areas. Two paraffin sections were cut from each case, one was placed on zinc-selenium slide and the other on glass slide. Thickness of all the tissue samples was 10 μm . The first slide was deparaffinized using xylol and alcohol and was used for FT-IR measurements. The second slide was stained with hematoxylin and eosin for histology review.

FT-IR microspectroscopy

FT-IR-measurements were performed in transmission mode. For the transmission measurement we used the FT-IR microscope with liquid nitrogen cooled MCT detector which is coupled to the FT-IR spectrometer (BRUKER EQUINOX model 55/S OPUS software). The microscope is also equipped with a CCD-camera for the visible range of the spectrum. Since the ordinary glass slides have strong absorption in the wavelength range of our interest, we used Zinc Selenide crystals, which are highly transparent to IR light. The pathologist examined each site using combined optical microscopy under standard histological conditions after staining. During each measurement, the measured areas of samples were typically a 100 μm diameter. Sixty different sites were measured for normal and similarly from malignant sections from ten different patients. The measured spectra cover the wavenumber range 600–4000 cm^{-1} in the mid-IR region. The spectrum was taken as an average of 128 scans to increase the signal to noise ratio. The spectral resolution was at 4 cm^{-1} . The baseline was corrected as follows. Initially, the spectrum was divided into 64 sections of equal size. Then the y-value minima of the spectrum were connected generating a curve which gives the best fit to the background. The spectrum was normalized to amide I peak at 1646 cm^{-1} after baseline correction for the entire spectrum.

Spectral analysis and fitting procedures

The band fitting analysis was performed using the software PEAKFIT (Version 4.0). The measured spectra were fitted using a standard Gaussian peak shape satisfying the following relationship:

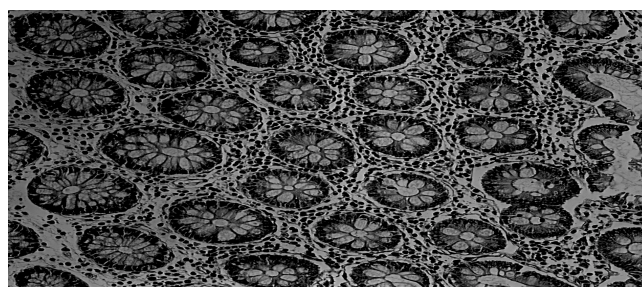
$$I(\nu) = I_0 \exp\left[-\frac{1}{2} \left(\frac{\nu - \nu_0}{w}\right)^2\right] \quad (1)$$

Where, $I(\nu)$ represents the IR absorbance at wavenumber ν , ν_0 is the peak centroid and w is the width parameter (6,20). The full width at half maximum (FWHM) of the band Γ is calculated using the relation

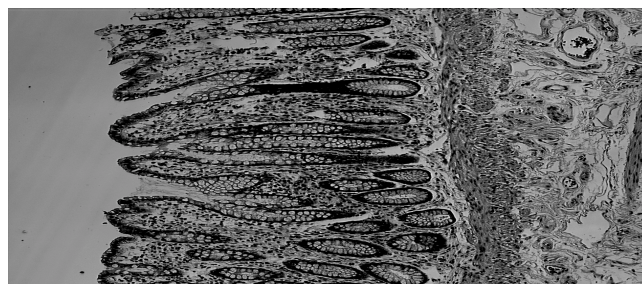
$$\Gamma = 2.36 w \text{ (cm}^{-1}\text{)} \quad (2)$$

With the enhanced resolution achieved due to high signal to noise ratio, the weak absorption bands can be resolved to a greater extent. Firstly, the second derivative spectrum was generated. The second derivative spectrum was used in identifying the hidden peaks. Secondly, the peaks obtained from the second derivative spectrum were fitted allowing the centroids, widths and absorption amplitudes to vary in the process. A linear function was used to fit the small residual background. The above analysis yielded reliable fits for the entire region of the spectra. Integrated areas were calculated (using OPUS software) to quantify the metabolites for normal and malignant tissue samples.

a



b



c

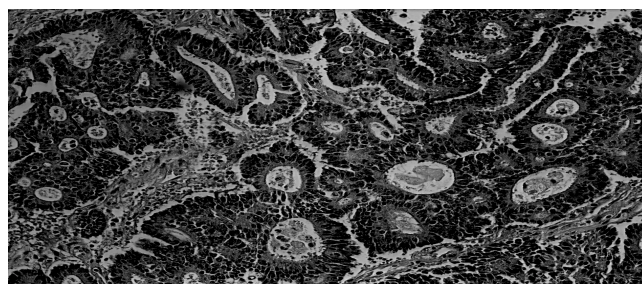


Fig. 1 Histological image of formalin fixed normal human colonic tissue stained with hematoxylin-teosin (a) cross section (b) lateral section (c) cross section of colonic cancerous tissue.

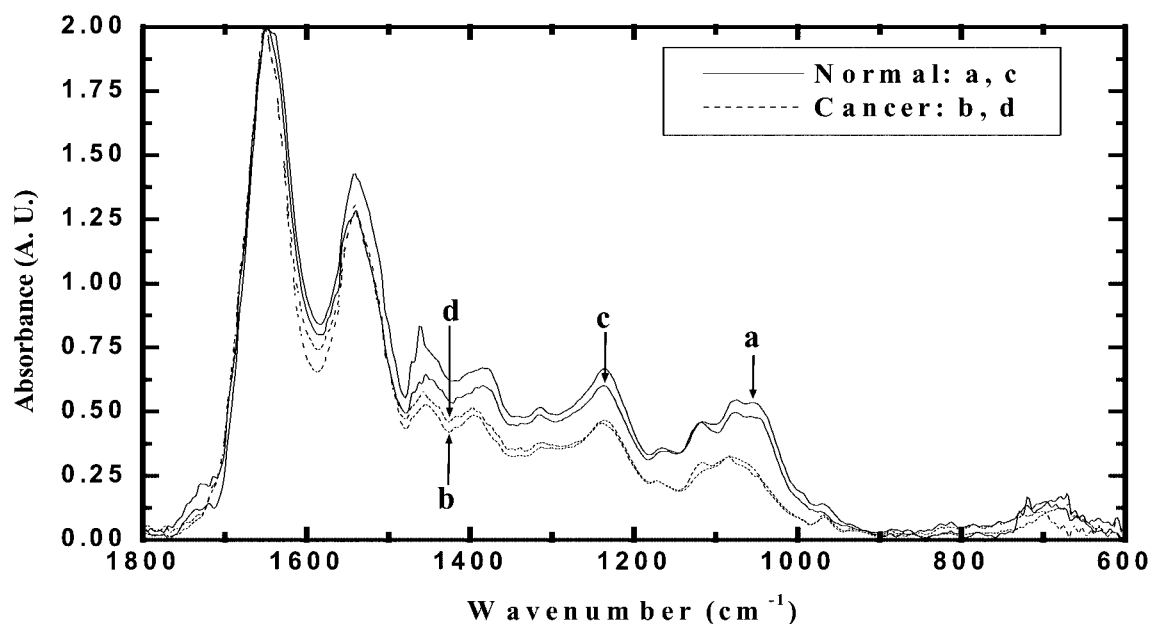


Fig. 2 Infrared microspectroscopy in the region 600–1800 cm^{-1} of normal (solid lines) and cancerous human colonic tissue (dashed lines) from two patients.

Linear discriminant analysis

To fully evaluate the performance of the proposed method, linear discriminant analysis (LDA) (14) was employed using MATLAB (Version 5.3, Math Works). 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. Fifty percent of each set was employed for training and the remainder for test. In addition, the validation experiment was repeated 100 times, with the same input features but with different sets of randomly selected training and test sets, and the results were averaged.

RESULTS

Fig. 1a presents histological cross section image of formalin-fixed human normal colonic tissues. The thin microvascular vessels pass vertically between crypts in the normal lamina propria. The normal colonic mucosa (Fig. 1b) is made up of epithelial lined crypts that are surrounded by lamina propria. The histological section is oriented in such a way that simple columnar surface epithelium facing the lumen is at the left of the Fig. and the lamina propria in which they are situated at the right of the Fig. The mucosal crypts are parallel to one another and unbranched. The adenomatous epithelium preserves mucus production-goblet cells. In this field there is a marked decrease in mucus production. There is focal loss of nuclear polarity, and an increase in nucleocytoplasmic ratio. Fig. 1c gives a low power view of adenocarcinoma, a malignant epithelial tumor. The gland shows cribriform

pattern, with loss of mucin production and foci of necrotic debris. The adenocarcinoma glands (grade II) are irregular and torous.

Fig. 2 shows typical infrared absorption spectra of normal (lines a,c) and cancerous (lines b,d) human intestinal tissues of two patients in the spectral range 600–1800 cm^{-1} . The absorption due to normal tissue was higher than cancerous types in this entire region of the spectrum. This was true for the other eight patients. No significant

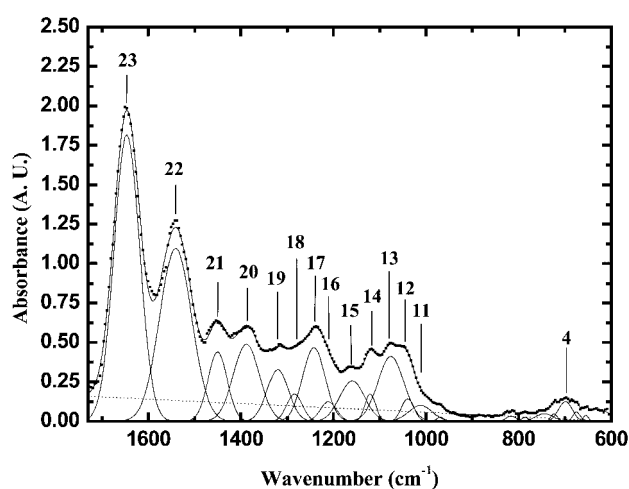
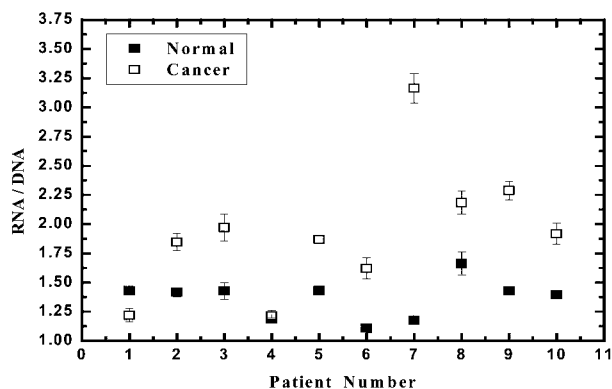
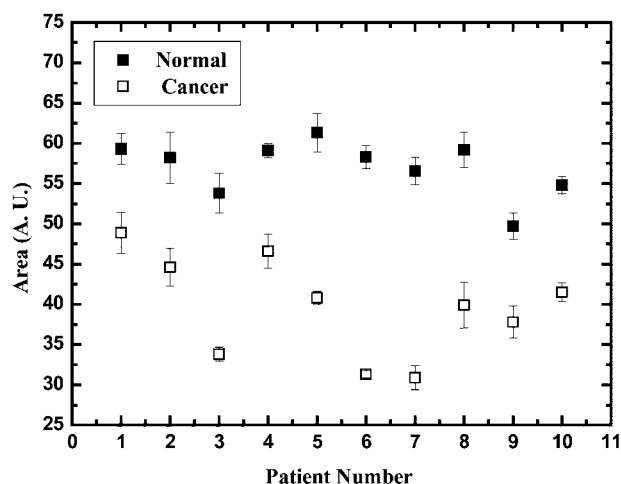
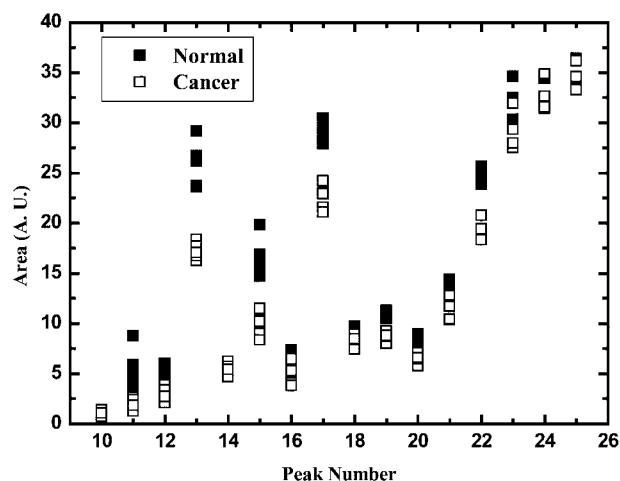


Fig. 3 Typical band fitting analysis in the wavenumber region 1730–600 cm^{-1} for patient number 8.



frequency shifts were observed between normal and cancerous tissues in the entire region ($600\text{--}4000\text{ cm}^{-1}$).

Spectral analysis and band fitting performed for the region $600\text{--}1730\text{ cm}^{-1}$ in the present work (shown for patient number 8) is shown in Fig. 3. The same fitting procedure has been used for the normal and the malignant sections.

Fig. 4 displays the analytical area for the different peaks between $950\text{--}1600\text{ cm}^{-1}$ deduced from the fitting analysis in the case of patient number 8. Out of 16 peaks shown in Fig. 4, the bands for phosphate (peaks 13 and 17, at 1075 and 1243 cm^{-1} due to phosphate group symmetric and asymmetric stretching vibrations respectively) show remarkable differences between normal and cancerous types. The analytical areas under the phosphate absorption were calculated for normal and malignant tissues for all the patients and they are presented in Fig. 5. The Fig. gives a clear picture of the variation in the phosphate content for normal and malignant tissues from all ten patients. In all the patients, the phosphate content of normal tissue was higher than the malignant tissues. The error bar was calculated for each sample (normal or malignant) separately for all the patients reported in this article.

An enhanced intensity ratio $I(1121\text{ cm}^{-1})/I(1020\text{ cm}^{-1})$ from normal to malignant is evident in literature spectra from several different tissues (7). This is an index of cellular RNA/DNA ratio after subtraction of overlapping absorbances. The results of our analysis are given in the Fig. 6. Except for the cases of patient numbers 1 and 4, the RNA/DNA ratio was higher for malignant tissues than the normal samples (by at least 70%).

Fig. 7 shows the higher wavenumber region between $2600\text{--}3800\text{ cm}^{-1}$ for normal and malignant tissues for two patients. Cholesterol, phospholipids and creatine are the three essential cellular metabolites, which absorb between $2800\text{--}3500\text{ cm}^{-1}$. Here also, the magnitude of normal tissue was higher than the cancerous types in all ten patients. Since there are symmetric and asymmetric

Fig. 4 (top) Analytical areas of the absorption bands observed in the region $950\text{--}1580\text{ cm}^{-1}$ for normal and cancer for patient number 8.

Fig. 5 (middle) Summed analytical areas of the phosphate bands labelled 13 (symmetric) and 17 (asymmetric) in Fig. 4. The filled and open symbols are normal and cancerous, respectively.

Fig. 6 (bottom) The intensity ratio at $1121/1020$ is presented as RNA/DNA for all the ten patients. The filled and open symbols are normal and cancerous samples, respectively.

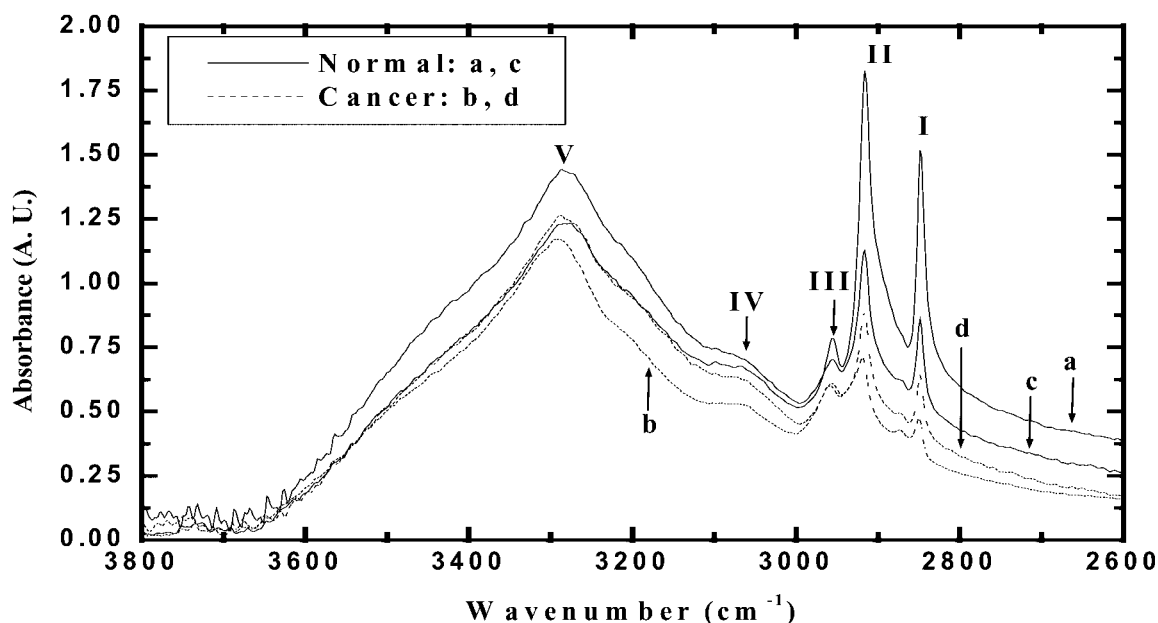
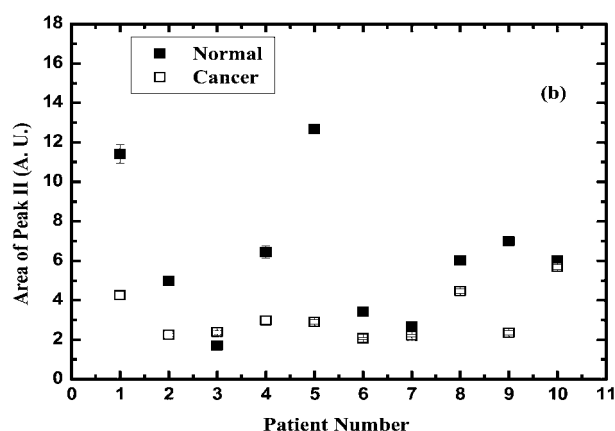
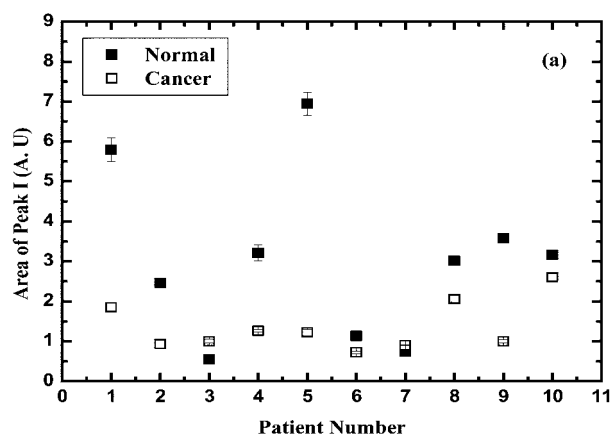


Fig. 7 Infrared microspectroscopy in the region 2600–3800 cm^{-1} of normal (solid lines) and cancerous human colonic tissue (dashed lines) from two patients. The wavenumbers of peaks I and II are 2848 and 2916 cm^{-1} , respectively.



vibrations due to water absorption in the region between 3200–3550 cm^{-1} , hence this region is not considered for analysis. The integrated areas (for peaks I and II in Fig. 7) at 2848 cm^{-1} and 2916 cm^{-1} for all the ten patients are presented in Fig. 8a–8b. The area for normal tissue was higher than cancer types in most of the cases.

The bands at 1025 and 1045 cm^{-1} in IR spectra are responsible for the vibrational modes of $-\text{CH}_2\text{OH}$ groups and the C–O stretching coupled with C–O bending of the C–OH groups of carbohydrates (includes glucose, fructose and glycogen, etc.) (24). The ratio of areas of the bands at 1045/1545 gives an estimate of the carbohydrate levels, which are shown in Fig. 9. Our results indicated that carbohydrate (including glycogen) levels were reduced in cancerous tissues in comparison to normals. This is in agreement with the decrease in phosphate levels presented in Fig. 5. It is surprising that the carbohydrate (glycogen) levels decrease in normal tissue relative to the cancerous type in the case of rectal region of the intestine (28), for which the reasons are not clearly understood and possibly it might arise from the different biological samples in the two cases.

Fig. 8 **a)** Integrated area of peak I (2848 cm^{-1}) for all ten patients. The filled and open symbols are normal and cancerous, respectively. **b)** Integrated area of peak II (2916 cm^{-1}) for all ten patients.

DISCUSSION

The informative PO_2^- symmetrical and asymmetrical stretching vibrations, which occur between $1000\text{--}1300\text{ cm}^{-1}$, provide clues to qualitative and quantitative changes for phosphate containing compounds. In our study, the intensity of these band structures for normal tissue was higher than cancerous types (as illustrated by bands 13 and 17 in Fig. 4 for patient number 8) in all ten patients. Mantsch and co-workers cautioned that in some studies the spectral differences observed between normal and malignant tissues could be due to collagen content (15). The examination of measured areas of the tissue sample in all the cases confirmed that the interference due to collagen was very minimal and hence our spectral changes are not due to collagen content. Also to

investigate the interference due to mucins layer present in the colonic tissue, the measurements were taken at different aperture values. Our results showed that the intensity changes at different values were not significant and the contribution due to mucins layer to the observed spectra was negligible. In the previous study reported by Rigas (27) the malignant tissues displayed decreased intensity of the $\nu_{\text{as}} \text{PO}_2^-$ band and increased intensity of the $\nu_{\text{sy}} \text{PO}_2^-$ band, when compared to normal (for colon cancer) tissues. They also reported that the intensity of these peaks varied from patient to patient and also within the sample. In our study, such discrepancies were not found, possibly the regions of colon and rectal may behave differently. Also, when the analytical areas for these two bands were summed up, the same trend was repeated (Fig. 5). The analysis of phosphate (symmetric and asymmetric) bands has clearly shown that the total phosphate content is significantly higher in normal tissues than malignant types.

Our results showed that in eight out of ten patients, the RNA/DNA ratio (Fig. 6) increased from normal to cancerous stage. These results correlate well with reports available in the literature. The region between $2800\text{--}3500\text{ cm}^{-1}$ is due to strong absorption of CH_2 , CH_3 stretching vibrations of phospholipids, cholesterol and creatine. Creatine and cyclocreatine have been shown to inhibit the growth of a variety of human and murine tumors. Antiproliferative effect of creatine is shown to be effective in mice carrying a human colon adenocarcinoma (LS 174T) (18). As in the case of phosphate bands, the intensity of higher wavenumber region for normal tissue was higher than cancer. Our results indicate that the creatine levels may be lower in cancer types compared to normal ones in accordance with the above studies.

The preliminary results of LDA are highly encouraging in discriminating normal from malignant cells. A summary of the different feature sets is shown in Table 1. The probabilities of classification using seven sets of features for normal and cancer tissues are shown in Table 2. The best results were obtained for set 1 (phosphate bands labelled 13 and 17, Table 1) with a success rate of 86.2% and 91.6% for normal and cancer tissue, respectively. Although the success rate of the LDA based classifier is high, a careful analysis of Table 2 reveals that about 8.4% of the cancer cases might be classified as normal while up to 13.8% of the normal cases might be classified as cancer.

Our FT-IR investigation on two types of colon tissues for ten patients showed that the levels of vital cellular

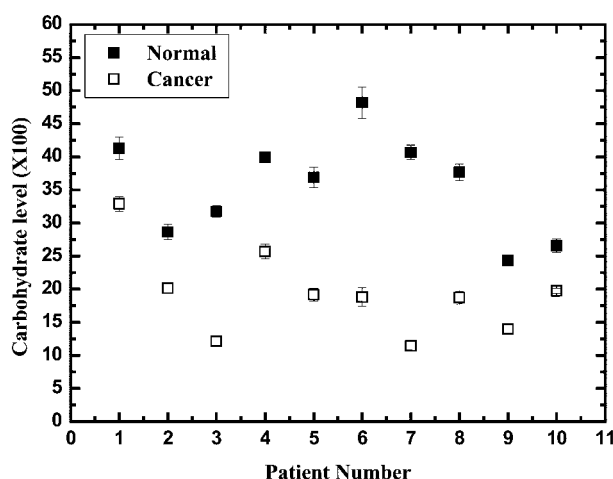


Fig. 9 The intensity ratio at $(1045/1545) \times 100$ for all patients. Filled and open symbols represent normal and cancerous, respectively.

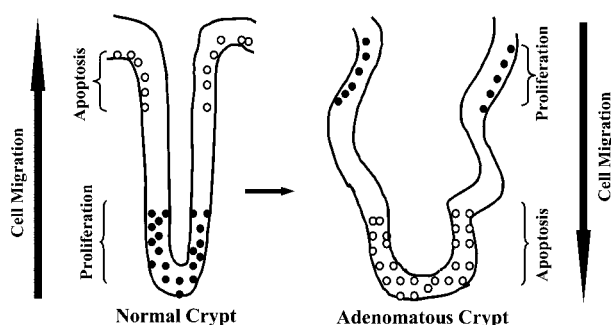


Fig. 10 The Schematic representation of the normal (left) and adenomatous crypt (right) showing cell migration and its corresponding living status. Filled and open circles represent the cells in exponentially growing and apoptotic stages, respectively.

metabolites decrease in cancer relative to the controls. These results are surprising since other cancer forms e.g. sarcoma (10), breast cancer (11) and skin cancer (29) show an increase in the phosphate concentration relative to the normal tissues. Colonic adenocarcinoma shows an entirely different behavior. Our results can be explained by having a better understanding of histogenesis of colonic adenomas. Generally, in normal crypt, the epithelial cells proliferate in the bottom portion and move further with differentiation and finally exit ending up in apoptosis. The recent model (schematically shown in Fig. 10) on histogenesis of colonic adenomas given by Moss *et al.* (23) claims that in adenomas, the proliferation of cells is predominant at lumen and apoptosis at the base, a complete reversal of normal pattern. It is interesting to speculate that the drastic decrease in cellular contents for cancerous tissues (as shown by our results) can be correlated to the apoptotic stage of cancerous tissues. This explains why our results are opposite to the findings for other tissue types. Additional evidences are necessary to substantiate our results by having more microscopic FT-IR data on samples from different sites of the adenomatous crypt in patients.

CONCLUSIONS

The absorbance for normal cells was higher than the malignant in all ten patients. The decrease in phosphate content was significant in malignant cells. The RNA/DNA ratio was higher for malignant tissue samples which can be a good biological marker. Also, probable decrease in creatine levels for malignant cells could be one of the risk factors as reported in vitro studies. The decrease in carbohydrate levels in malignant tissues may be the possible reason for the reduction in cell metabolites. Also, this accounts for higher intensity for normal tissues than the malignant samples in the entire region of the spectrum. Multivariate analysis showed reasonably good classification between normal and malignant samples. Our study with ten samples of normal and malignant colonic tissues has clearly shown that FT-IR microscopy can be developed for the purpose of diagnosis. However large database is essential to turn this methodology into novel diagnostic technique for colon cancer using FT-IR. Studies with larger number of human samples and use of state of the art imaging techniques may improve the results in the early diagnosis of colon cancer in the future.

Table 1 Feature combinations of the linear discriminant analysis

Feature Description	Vector size	Feature Identification
Phosphate bands labelled 13 and 17	2	1
Integrated area of peaks I (2848 cm ⁻¹) and II (2916 cm ⁻¹)	2	2
The intensity ratio at I(1121) / I(1020) and I(1045) / I(1545)	2	3
The intensity ratio at I(1045) / I(1545) and summed analytic areas of the phosphate bands labelled 13 and 17	2	4
Phosphate bands labelled 13 and 17 and the intensity ratio at I(1045) / I(1545)	3	5
Phosphate bands labelled 13 and 17 and RNA	3	6
Phosphate bands labelled 13 and 17 and the intensity ratio at I(1121) / I(1020)	3	7

Table 2 FT-IR assessment for Cancer: the percentage of correct and incorrect test diagnoses

Feature Identification	Normal as Normal	Cancer as Cancer	Normal as Cancer	Cancer as Normal	Total*
1	86.2	91.6	13.8	8.4	88.9
2	93.2	55.4	6.8	44.6	74.3
3	84.4	84.4	15.6	15.6	84.4
4	84.6	89.6	15.4	10.4	87.1
5	80.4	78.2	19.6	21.8	79.3
6	77.0	90.8	23.0	9.2	83.9
7	83.2	90.2	16.8	9.8	86.7

* Total is the average of positive identification for both normal and cancer types (columns 2 and 3).

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