Discrimination of Patients with Microsatellite Instability Colon Cancer using \(^1\)H HR MAS MR Spectroscopy and Chemometric Analysis

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The primary aim of this study was to analyze human colon cancer and normal adjacent tissue using \(^1\)H HR MAS MR spectroscopy and chemometric analyses, evaluating possible biomarkers for colon cancer. The secondary aim was to investigate metabolic profiles of tissue samples (\(n = 63, 31\) patients) with microsatellite instability (MSI-H) compared to microsatellite stable (MSS) colon tissue. Our hypothesis was that this method may provide an alternative to MSI genotyping. Cancer samples were clearly separated from normal adjacent mucosa by 100% accuracy. Several metabolites such as lactate, taurine, glycine, myo-inositol, scyllo-inositol, phosphocholine (PC), glycerophosphocholine (GPC), creatine, and glucose were identified as potential biomarkers for cancer detection. Adenomas (\(n = 4\)) were also separated from cancer and normal samples mainly based on higher GPC and PC levels. Interestingly, metabolic differences in normal colon mucosa between MSI-H and MSS patients were observed. MSI status was validated with 80% accuracy with a sensitivity and specificity of 79% and 82%, respectively, including both cancer and normal samples. The metabolic differences between MSI-H and MSS may be very interesting in the early detection of cancer development and of high clinical importance in the work of improving diagnosis and characterization of colon cancer.

Keywords: metabolomics • metabolites • promoter MLH1 methylation • MSI • MSS • PCA • PLSDA

Introduction

Colorectal cancer (CRC) is one of the most common malignancies and leading cause of cancer-related deaths. In Norway, CRC is the second most frequent cancer and the incidence is higher compared to the U.S., where CRC is the third most commonly diagnosed type of cancer, accounting for 10% of estimated new cases among leading cancer types. The incidence of CRC in Norway has been highly increasing the last 50 years, and today the incidence is about the highest among European countries.

Genetic instability can differ from whole chromosomal aberrations to small differences in a few base pairs, causing several changes to the phenotypical traits of the cell. In colorectal cancer, genetic instability is usually classified into three pathways including chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylation phenotype (CIMP). MSI is referred to as abnormally long or short repetitive DNA sequences, microsatellites, which are caused by defects in the DNA mismatch repair (MMR) system. The majority of patients with hereditary nonpolyposis colorectal cancer (HNPPC/Lynch syndrome) have high-frequency microsatellite instability (MSI-H) caused by an inherited mutation in one of the MMR-genes, and about 15% of sporadic colorectal cancers are characterized by MSI-H caused by epigenetic changes. MSI status is important as a screening tool for HNPPC/Lynch syndrome, as a prognostic marker and as a predictor of response to therapy. CRC patients with MSI seem to have a better prognosis and survival compared to those who are microsatellite stable (MSS). One study found that patients with MSI-H tumors do not respond to a certain type of adjuvant chemotherapy (fluorouracil) for colon cancer, but the predictive value of chemosensitivity is controversial. Therefore, a better understanding of tumor biology is important for optimizing treatment strategies and for the implementation of MSI analyses in a clinical setting.
Discrimination of Patients with MSI Colon Cancer

MSI in sporadic colorectal cancer usually arises from epigenetic silencing of the DNA mismatch repair gene MLH1, which is caused by methylation of promoter sequences of the gene. Epigenetic silencing, the third pathway of genetic instability, will not change the DNA sequence but affects the gene function by causing deficiency in protein expression. CRC patients with MLH1 methylation causing MSI-H tumors are found to have a better cancer-specific survival compared to patients with MSS tumors. The classification of MSI-H and MSS is therefore of clinical importance in the assessment of prognosis. New methods are therefore needed to elucidate biological mechanisms within the MSI pathway.

High resolution proton magic angle spinning (1H HR MAS) is an ex vivo method providing detailed information on metabolic composition (metabolic profiles) of several types of intact cancer tissue. Metabolic profiling of CRC using 1H HR MAS has recently shown promising results for understanding CRC and for optimizing clinical management strategies. A few studies have presented that metabolic profiles of colorectal cancer and normal mucosa are clearly discriminated analyzed by 1H HR MAS. Altered metabolites in CRC tissue were associated with expected metabolic perturbation such as elevated tissue glycolysis, hypoxia, nucleotide biosynthesis, lipid metabolism, inflammation, and steroid metabolism. Correlating metabolic profiles to the pathways of genetic instability and clinical parameters may provide a better understanding of colon cancer development using 1H HR MAS MR spectroscopy.

MR spectra are highly multivariate data sets requiring sophisticated statistical analysis, chemometric approaches, to provide information about the metabolic status of a tissue sample. One of the most common methods is principal component analysis (PCA), by which linear combinations of the variables explains the variance structure in the data set. This can reveal hidden relationships, patterns or grouping of the multivariate MR spectral data. A supervised method such as partial least-squares regression discriminant analysis (PLSDA) attempts to discriminate between groups, for example, cancer and normal tissue, and where the relationship between two matrices $x$ (MR spectra) and $y$ (properties related to each spectrum) is modeled. These chemometric analyses are important tools for model building and classification of MR data.

The primary aim of this study was to characterize the metabolic profile of human colon tissue using 1H HR MAS and to evaluate possible biomarkers for colon cancer. The secondary aim was to investigate metabolic profiles of samples with MSI-H, caused by promoter MLH1 methylation, compared to MSS. Our hypothesis was that metabolite profiling using 1H HR MAS and chemometric analyses may provide a reliable rapid alternative or additional test to MSI genotyping. The hypothesis was validated using blind samples which had not been used earlier in the model building.

**Material and Methods**

**Patients and Tissue Collection.** Fresh frozen colon tissue samples ($n = 63$) from two hospitals in Norway were obtained from 31 newly diagnosed cancer patients (mean age: 73 years (range: 48–93), 16 women and 15 men). Two samples were collected from each patient; one from the tumor area and the other from normal mucosa. Twenty-eight samples were collected from patients diagnosed with colon adenocarcinoma and 4 samples from patients diagnosed with adenomas. One patient had two malignant tumors in the operation species, and both were included in the analyses. Thirty-one samples of normal mucosa were collected. Eighteen cancer samples were MSI-H and 14 cancer samples were MSS. Normal adjacent tissue was also labeled MSI-H if the patient had an adjacent tumor that was MSI-H. Including both cancer, adenomas and normal mucosa, a total of 35 MSI-H samples and 28 MSS samples were analyzed. Two of the MSI-H tumors had no MLH1 promoter methylation and was therefore most likely HPNCC/Lynch syndrome tumors. Clinical patient data are shown in Table 1. The study was approved by the Regional Committee for Medical and Health Research Ethics, and written informed consent was obtained from all contributing patients.

Preoperatively, broad-spectrum antibiotics and low-molecular-weight heparin had routinely been administered to the patients. In the majority of cases, the patients had been given mechanical laxatives preoperatively. Tissue samples were collected immediately during surgery and frozen at $-80^\circ$C.

**1H HR MAS Spectroscopy and Data Processing.** A phosphate-buffered saline (PBS, 3 μL) based on deuterium oxide (D2O) containing 4.5 mM TSP (sodium-3′-trimethylsilylpropionate-2,2,3,3-d4) and 20 mM formate was added to disposable Kel-F HR MAS inserts (30 μL, Bruker, Germany). The colon tissue (weight; cancer: 14.4 ± 2.6 mg and normal: 15.1 ± 4.2 mg) was added to the HR MAS insert using a sterile disposable biopsy punch with plunger (2 and 1.5 mm, Miltex Gmbh, Germany), and the insert was placed into the 4 mm zirconium HR MAS rotor. 1H HR MAS was performed on a 14.1 T Bruker Avance DRX spectrometer equipped with a 4-mm 1H/13C MAS probe. Spectra were acquired at 4°C with a spin rate at 5 kHz. A Carr-Purcell-Meiboom-Gill (CPMG) spin echo sequence [90° – (τ–180°–τ)] with an effective echo time of 272 ms was acquired to suppress signals from overlapping lipids and macromolecules. One hundred and twenty-eight scans over a spectral region of 10 kHz were collected into 64k points, giving an acquisition time of 3.27 s. An exponential line broadening of 0.30 Hz was applied to the raw data before Fourier transformation. The spectra were chemically shift referenced to the lactate peak (left peak in the doublet) at 1.33 ppm, and a linear baseline correction was applied. MR data points were included in the analyses.
reduced from 64k to 16k for chemometric analysis. For assignment purposes, total correlation spectroscopy (TOCSY) was performed on some of the tissue samples.

**Histopathology.** After HR MAS analysis, the tissue samples were fixed in 10% formalin, embedded in paraffin, sectioned (3 µm) and stained with HES (hematoxylin, erythrosine and saffron) for pathology reading. The sections were deparaffinised in xylene for 10 min two times and subsequently rehydrated in decreasing concentrations of alcohol. An experienced pathologist examined the tissue slides and provided percent cancer tissue content (desmoplastic stroma not included) and information about necrosis and inflammatory responses.

**Analyses for MSI and Promoter MLH1 Methylation.** Analyses of MSI and MLH1 methylation were performed as described by Tranø et al.21

**Chemometric Analysis.** The spectral region between 1.4 and 4.8 ppm was transferred to ASCII format and used for multivariate analysis. Contaminated areas due to ethanol and polyethylene glycol (from preoperative bowel preparation) (3.63–3.73 ppm) and the dominating peak from acetate (1.90–1.94 ppm) were removed from the data set, resulting in a total of 3166 variables. The final data were mean normalized and mean centered before further analysis by principal component analysis (PCA). The spectral data (n = 63) were also used to build models for cancer and normal tissue, MSI-H and MSS samples and gender by using partial least-squares discriminant analysis (PLSDA). The relationship between age and the spectral data was modeled using partial least-squares regression (PLS). One cancer sample was excluded from the prediction model of cancer and normal because of a cancer content <1%. The MR spectra from the cancer samples (n = 27) were also analyzed with respect to T-stage, differentiation (medium/low), Dukes stage (A, B, C, D) and anatomical site of cancer (right colon defined as proximal to the splenic flexure) using PLSDA. Number of samples used for each test differed due to availability of clinical data (see Table 1). The number of latent variables (LVs) used for building the models was determined by finding the LV where the total residual y-variance and root-mean-square error of prediction were minimized. Full cross-validation was applied. All statistical analyses were performed using PLS_Toolbox 5.2.2 for Matlab (Eigenvector Research, Wenatchee, WA). To investigate robustness of the models for prediction of cancer and MSI, separate sample sets for model building and validation were established based on unsupervised sample selection22 of all the spectral data (n = 63). The Kennard Stone algorithm selects samples that are furthest away from each other in the data set one by one. The samples with the maximum distance (variance) were used for model construction (n = 38), and the Kennard-Stone algorithm assured representative samples for the validation (n = 25). The training model was based on full cross validation. The validation samples were kept outside all model training. To further validate the PLSDA results, additional permutation tests were performed 10 000 times both for the cancer and normal model and for the MSI-H and MSS model.

**Results**

**Discrimination between Cancer, Adenoma and Normal Mucosa.** Representative 1H HR MAS spectra (CPMG sequence, selected region 2.8–4.3 ppm) from colon cancer tissue (adenocarcinoma in the right colon) and normal mucosa from the same patient are shown in Figure 1a. By visual comparison, the spectra show a relative increase in lactate, glycine and taurine resonances in the colon cancer tissue compared to normal tissue, and an obvious decrease is seen in myo-inositol and glycerophosphocholine (GPC) resonances in the cancer tissue. Histopathological examination of the samples used in the 1H HR MAS analyses showed a cancer content of 52% on average (range: 7–90%).

The PCA score plot based on all spectra (n = 63) points out the largest variation among the colon spectra, and when plotting PC1 against PC2 (describing 50% of total variation), there was a clear discrimination of cancer tissue samples from both adenomas and normal samples (Figure 2a). The four adenomas were also clearly separated from the normal samples and there was no overlap between any of the three groups. The
four patients diagnosed with adenomas were separated from the normal samples by PC1 but also from the cancer samples by PC2. The loadings for the PCA model (not shown) indicated a considerably higher relative concentration of phosphocholine (PC) in three of the adenomas (discriminated by PC1, 31% of the variance) compared to both cancer and normal tissue and a higher relative concentration of GPC (discriminated by PC2, 19% of the variance) in 1 of the 4 adenomas. Another interesting observation in the PCA score plot was that the two patients most likely having the hereditary type of MSI cancer (HNPCC/Lynch syndrome) had scores appearing close to the normal samples (Figure 2a). Tumors from these two patients had no methylation of the \textit{MLH1} promoter.

To demonstrate the differences between cancer and normal colon HR MAS spectra, the adenomas were removed from the data set and PLSDA was performed on all the cancer and normal samples (\(n = 58\)). The PLSDA model was based on 3 LVs for describing the total residual \(y\)-variance. The score plot of LV1 against LV2 (explaining 45% \(x\)-variance and 83% \(y\)-variance) showed a clear separation of the cancer samples from the normal samples. The cross-validated correlation between the true class and the class predicted is 0.89 (\(r_{\text{calibration}} = 0.94\), Figure 2b). The cancer samples had a more scattered distribution than the normal samples in the PLSDA score plot (Figure 2b). This is also shown in the results listed in Figure 2a. The loadings for LV1 (Figure 2c, explaining 24% \(x\)-variance and 79% \(y\)-variance) showed metabolic alteration in several metabolites of cancer tissue compared to normal colon mucosa.

\begin{table}[h]
  \centering
  \begin{tabular}{|l|c|c|c|c|}
    \hline
    & all samples & all samples & cancer & normal \\
    & without & with & (\(n = 27\)) & (\(n = 31\)) \\
    adenomas & adenomas & (\(n = 39\)) & (\(n = 63\)) & \(0.94/0.89^c\) \(0.89/0.79\) \\
    \hline
    Cancer/normal & 0.92/0.82 & 0.94/0.89 & & \(d\) \(d\) \\
    MSI-H/MSS & 0.81/0.50 & 0.86/0.58 & & \(d\) \(d\) \\
    Age & 0.66/0.43 & 0.67/0.45 & 0.85/0.56 & 0.83/0.59 \(d\) \(d\) \\
    Anatomical site & & & & 0.46/0.21 \(d\) \(d\) \\
    (left/right) & & & & \\
    Gender & & & & 0.65/0.32 \(d\) \(d\) \\
    Dukes classification & & & & \\
    (A,B,C,D) & & & & \\
    T-stage & & & & 0.79/0.53 \(d\) \(d\) \\
    (T1, T2, T3, T4) & & & & \\
    Lymph node status & & & & \\
    (Nx, N1, N2) & & & & \\
  \end{tabular}
  \caption{Result from PLSDA of All the Tested Parameters\(^a\)}
  \end{table}

\(^a\) Correlation coefficient for both the calibration and validation (in bold) are listed. \(^b\) \(n = 58\). \(^c\) \(n = 62\). \(^d\) Not able to build a model based on the data, no reduction in residual variance.
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Figure 3. (a) PLSDA score plot of LV1 and LV2 (explaining 41% x-variance and 44% y-variance) shows separation of metabolic profiles from patients with MSI-H (n = 35) from MSS (n = 28) in LV2. (b) LV2 loadings plot (explaining 13% x-variance and 21% y-variance) shows metabolic alteration in several metabolites of MSI-H samples compared to MSS samples.

PLSDA with a correlation of 0.58 ($r_{\text{calibration}} = 0.86$, Figure 3a). The PLSDA model was based on 5 LVs for describing the total residual y-variance, and the model included all samples ($n = 63$). Results from PLSDA performed only for cancer and normal samples and with or without the adenomas are shown in Table 2. Using only the cancer samples, no reduction was found in residual variance and therefore no model was built. In the normal samples, there was a correlation of 0.53 ($r_{\text{calibration}} = 0.79$), showing that the correlation using all samples is in reality found in the normal tissue material. The score plot from the PLSDA of LV1 and LV2 (Figure 3a, $n = 63$, explaining 41% x-variance and 44% y-variance) show a fair separation between the MSS and the MSI-H samples, mainly due to LV2. The adenomas ($n = 4$) have the highest score for LV1 and cause a higher dispersion among the MSS samples, and the remaining MSS and MSI-H are mainly separated on LV2. The loadings for LV2 (Figure 3b, explaining 13% x-variance and 21% y-variance) show the metabolites responsible for the observed separation between MSI-H and MSS. MSI-H samples have a relative higher concentration of lactate, glycine, taurine, scyllo-inositol, PC, choline and creatine than MSS samples, and a lower relative concentration of $\beta$-glucose, myo-inositol and GPC.

The model for prediction of MSI was based on cross validated data from 38 samples chosen by Kennard Stone, and the 25 validation samples were classified 80% correctly (20/25 samples) as MSI-H and MSS. The model provided a sensitivity and specificity of 79% and 82%, respectively. Permutation testing ($n = 10,000$) showed that the prediction results were significantly different from random ($p = 0.005$).

Discussion

Marked differences in metabolic profiles of colon cancer and normal mucosa, validated with 100% accuracy, were found using $^1$H HR MAS and chemometric analyses (PCA, PLSDA; $r = 0.89$). This is in compliance with the earlier studies using MR metabolomics in analyses of CRC tissues.$^{14-16}$ Colon tissue samples being MSI-H were predicted with 80% accuracy (79% sensitivity, 82% specificity) using blinded validation data, and permutation testing revealed that the prediction for MSI-H was significantly different than random ($p = 0.005$). To our knowledge, this is the first study to combine HR MAS MR data to the genetic marker MSI caused by promoter methylation of MLH1 in colon cancer tissue. The fact that the correlation was mainly found in the normal mucosa (Table 2) is interesting when considering that metabolic difference related to MSI-H may act as early markers for colon cancer. These observations and previous studies using other techniques$^{23,24}$ show that gene-specific promoter (hyper-)methylation and separation of the MSI- and MSS-pathway is an early event in tumorigenesis of CRC.

Discrimination between Cancer, Adenoma and Normal Mucosa. The validation model for prediction of cancer and normal mucosa of 100% accuracy confirms previous results which state that HR MAS analysis has a high sensitivity and specificity for discrimination of cancer and normal tissue.$^{14-16}$ Additionally, the specific metabolic differences (Figure 2c) between cancer and normal tissue were in compliance with these studies. Increased levels of lactate and decreased levels of glucose in cancer tissue (Figure 2c) indicates possible mechanisms such as increased glycolytic flux due to hypoxia and ischemia in the tumor area,$^{25}$ or the Warburg effect where an increased uptake of glucose and conversion to lactate is seen in tumor cells even in high oxygen conditions.$^{26}$ Higher levels of the amino acid glycine found in this study can also be explained by glycolytic variation due to transformation from the glycolytic intermediate 3-phosphoglycerate in CRC specimens, as suggested by Chan et al.$^{14}$ However, sarcosine which is a N-methyl derivate of glycine, is recently identified by mass spectrometry as a differential metabolite that is highly increased during prostate cancer progression to metastasis, and which can be detected in urine.$^{27}$ This pathway could be worth investigating also in colon cancer.

The choline-containing compounds (ChoCC) PC and free choline are important constituents in cell membranes, and several MR studies of different organs have confirmed elevated levels of these metabolites in malignant tumors.$^{28}$ Increased levels of PC and free choline were also found in colon cancer specimens compared to samples from normal tissue in the present study. A previous study by Nakagami et al. showed that the choline kinase activity is increased in colon cancer tissue with following elevation of PC,$^{29}$ and they suggested that choline kinase may play a role in growth promotion or signal transduction in carcinogenesis. Increased levels of PC are also observed previously in CRC tissue.$^{14}$ Interestingly, GPC (a membrane breakdown product) was decreased in the colon cancer samples compared to normal samples in this study, and this is to our knowledge not previously documented in MR studies on human colon cancer tissue. It is stated that the
normal human gastrointestinal (GI) tract have relatively high levels of phospholipid intermediates, and cancer may disturb the highly efficient normal cell proliferation in the colon mucosa. Lower levels of GPC were however observed in biopsies from patients with active Ulcerative Colitis compared to normal controls, and Wang et al. have observed decreased GPC levels in the human colon compared to the small bowel. The latter study shows that accuracy in location of the GI tract is important when considering GPC levels. Other metabolites such as lactate, myo-inositol, scyllo-inositol and taurine are also found to differ along the GI tract. However, since only tissue from colon is included in this study, the issue on location along the GI tract can not be assessed.

Due to hypertonic conditions during digestion of food in the GI tract, osmotic regulation is important for balancing osmotic equilibrium between cells and the surrounding medium. This is achieved through maintaining a high intracellular content of osmolytes such as myo-inositol, scyllo-inositol and taurine. Normal colon mucosa have been documented to be rich in osmolytes such as myo-inositol and scyllo-inositol, which are important for regulating long-term hypo-osmotic/hyper-osmotic stress. In this study, we detected a decrease in both myo-inositol and scyllo-inositol in colon cancer tissue compared to normal tissue. This phenomenon is also described by the previous CRC MR studies; however, myo-inositol was not presented in the study by Chan et al. Taurine is another commonly found osmolyte, important for short-term hypo-osmotic stress. In the present study, taurine was increased in the colon cancer mucosa, which is previously described in MR studies. The observed osmolyte changes in colon cancer tissue (decreased levels of inositols and increased levels of taurine) may indicate imbalance in osmolyte function in cancer cells. Taurine has many functions but has also been implicated in the mechanism of cell shrinkage during apoptosis and may therefore be a potential marker of apoptosis, which is also found in human gliomas.

Adenomas (adenomatous polyps) can be precancerous lesions in the colon mucosa, and are therefore interesting for studying precancerous conditions. This study included only four adenomas, but the results show distinct differences in the metabolite profile compared to both cancer and normal mucosa, especially in the PC and GPC levels (plot not shown). The four adenomas are also observed with a large dispersion in the score plot. The results have to be confirmed with a larger amount of samples. The metabolic difference can be associated with different grades of dysplasia or the presence of infiltrating growth of a mucosal lesion. The metabolic difference observed in adenomas may be important information for the individual patient when accessing the risk of cancer development.

This study aimed to correlate colon HR MAS spectra to clinical parameters such as age, anatomical site, differentiation, gender, Dukes stage and lymph node status. No correlations were detected except to age (Table 2). Due to a low number of younger cancer patients (6 of 31 patients were below 60 years), a study including a higher number of CRC patients of different age groups will be necessary to assess whether metabolic differences are age dependent.

Discrimination between MSI-H and MSS. The validation model for prediction of MSI-H and MSS samples of 80% accuracy (sensitivity and specificity of 79% and 82%, respectively) indicates an evident difference between the MR spectra of these tissues. A difference between MSI-H and MSS is however not found when testing only the cancer samples, but the correlation in this study was detected among the normal samples (Table 2). This absence of correlation in cancer may be caused by extreme metabolic conditions in the tumor microenvironment due to mechanisms such as hypoxia and high proliferation rates. There is also evidence that hypomethylation occurs in cancer tissue and that the epigenetic alterations, not the genetic alterations, occur in normal tissue. Ramirez et al. observed an increase in methylation of promoter MLH1 in normal colon mucosa. This supports the phenomenon that associations found in neighboring mucosal tissues are not found in tumor tissue. MLH1 methylation has previously been classified as Type C methylation (cancer-specific), which is in contrast to our results on metabolic profiles and to prior studies on methylation. Metabolic alterations found in normal mucosal HR MAS spectra may describe an early state of cancer development that may occur prior to genetic alterations. These metabolic differences detected in normal cells without cancer phenotype may serve as early biomarkers for cancer development and progression. Histopathology after HR MAS confirmed that there was no cancer invasion in these normal tissue samples.

Although the separation between MSI-H and MSS samples in the score plot (Figure 3a) is not completely separated, it is possible to point out specific metabolites responsible for the separation (Figure 3b). The MSI-H tissue samples were characterized by increased relative concentrations in the majority of the metabolites; lactate, glycine, taurine, creatine, PC and free choline. GPC, myo-inositol and glucose were lower in the MSI-H samples compared to MSS. Comparing the loading profile obtained by the PLSDA of cancer and normal samples (Figure 2c), the MSI-H samples were separated from MSS samples on the same metabolic basis as cancer is separated from normal. This indicates that normal samples that have a neighboring tumor with the promoter MLH1 methylation causing MSI-H have the same metabolic changes observed in histopathologically confirmed cancer samples. These metabolic changes detected in histopathologically confirmed normal tissue is very interesting regarding new biomarkers for cancer development and potentially in patient prognostics.

In conclusion, considering the fast and cost-effective HR MAS analysis on intact tissue, the metabolic picture in normal neighboring mucosa may be an important additional analysis in improving clinical diagnosis and characterization of colon cancer. HR MAS analysis of normal adjacent mucosal tissue may provide an indication of early development of colon cancer.

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