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Evaluation of intestinal cancer by 1H HR-MAS NMR spectroscopy: A new diagnostic tool?

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ABSTRACT

Background: Previous studies have demonstrated that cancer cells harbor unique metabolic characteristics relative to healthy counterparts. The current study is a prospective ex-vivo HR-MAS NMR analysis of malignant colorectal cancer (CRC) tissue specimens and its corresponding benign tissues.
Objective: To assess the HR-MAS Spectroscope qualitatively & to analyze significant difference between the normal, benign and malignant intestinal mucosa.
Materials and Methods: Between November 2013and January 2016, 36 consecutive patients with confirmed CRC were recruited to a prospective observational study. Fresh tissue samples were obtained from center of tumor and 5 cm from tumor margin from surgical resection specimens. Samples were run in duplicate where tissue volume permitted to compensate for anticipated sample heterogeneity. Typically, the sample was packed into a 4 mm ZrO2 rotor of 50 μl capacity; a volume of 20μl of D2O having 0.03% TSP was used as a chemical shift reference. The sample-rotor-setup was then transferred into the HR-MAS NMR probe for analysis.
Results: A total of 36 spectra were acquired (center of tumor, n = 18; 5 cm from tumor margin, n = 18). The malignant clustering occurs due to increased Val (0.90ppm), Lac (1.34ppm), Ala(1.48ppm) levels of acetate (1.90ppm), glutamate (2.35ppm), taurine (3.23 ppm), choline containing compounds (3.20-3.22ppm), glycine (3.56ppm), lactate (4.12ppm) and α-H of Leu, Ileu, Val, Lys, Ala (3.76-3.79ppm . In addition unique metabolic profiles were observed for tumors of differing T-stage. The information gathered from clustering in PCA had highly suggested that malignancy induces metabolic perturbations at cellular levels.
Conclusions: HR-MAS NMR profiling demonstrates cancer-specific metabolic signatures in CRC and reveals metabolic differences between benign and malignant tumors. In addition, this approach reveals that tumor metabolism undergoes modification during local tumor advancement, offering potential in future staging and therapeutic approaches.

1. Introduction

Colorectal cancer (CRC) is already the third leading cause of cancer death in the world, and its incidence is steadily rising in developing nations. Also known as colorectal adenocarcinoma, CRC usually emerges from the glandular, epithelial cells of the large intestine. Worldwide, colorectal cancer (CRC) is the second most common malignancy and is a leading cause of cancer associated death in many developed countries. Among CRC, 65% of CRC are rectal cancer which is located in the lower end of colon.

The cancer arises when certain cells of the epithelium acquire a series of genetic or epigenetic mutations

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that confer on them a selective advantage.[1] With abnormally heightened replication and survival, these hyper-proliferative cells give rise to a benign adenoma, which may then evolve into carcinoma and metastasize over decades.

Colorectal cancer was the most increased cause of cancer death over a 25 year period, with a 4.8 fold increase observed in males and a 3.6 fold increase observed in females. Similarly, the incidence of cancer also increased by 43% in males and 32% in females.

Pre-symptomatic screening aims to detect early stage CRC or its precursor lesions for improved cancer specific survival and reduced treatment related morbidity.

Unfortunately, only about 37% of CRC remain localized at the time of diagnosis. Five year survival rate of rectal cancer patients is 93.5 % for stage I, 87.4% for stage II, 58.2% for stage III, and 8.1% for stage IV.[2]

The reason that result in late diagnosis and therapy as well as disappointing low survival rate include ineffective screening tools and guidelines, cancer detection at an advanced stage, limited survival achieved with palliative chemotherapy alone for patients with metastatic or unresectable disease. Therefore early and accurate diagnosis of rectal cancer is critical for patient survival and improving therapeutic options for different stages of rectal cancer.

Present diagnostic and screening methods of CRC, such as colonoscopy, flexible sigmoidoscopy, double contrast barium enema, virtual colonoscopy, fecal occult blood test, fecal based DNA test, and serum based protein markers such as carcinoembryonic antigen (CEA), have certain limitations and shortfalls in their own way.

Metabolomics is an emerging field of research downstream of transcriptomics, genomics, and proteomics, which mainly involves the multicomponent analysis of biological fluids, tissues and cell extracts. It is currently used as a model of research in many disciplines of medicine, including disease diagnosis[3,4] biomarker screening[5,6] nutritional intervention[7] and safety assessment of chemical.[8,9] Three powerful analytical techniques are commonly applied to assay and quantify metabolites, including liquid chromatography (LC) coupled with mass spectrometry (MS), gas chromatography MS (GC/MS) and nuclear magnetic resonance (NMR).[10] NMR has been used extensively since 1970s. It has some advantages over MS in metabolic application, including non-destructive analysis, the relative ease of sample preparation, the potential to identify a broad range of compounds and the capacity for the supply of structural information for unknown compounds.[11,12] Until now, only several NMR-based studies using patient colorectal cancer tissues have been reported.[1,13] However, the number of patient tissues in these studies was limited, which cannot provide accurate and comprehensive information of CRC metabolites. Moreover, discriminating metabolites involved in the different pathological stages of rectal cancer have not been investigated. Therefore, it will be valuable to perform metabolic profiling of human rectal cancer tissues in aiding molecular diagnosis and providing novel insights into rectal cancer.

We in this present study investigated the difference between normal and cancerous colorectal tissue using HR-MAS spectroscopy.

2. Aims and Objectives

1. To obtained malignant, benign, and normal intestinal tissue from patients/subject.
2. To assess the HR-MAS Spectroscope qualitatively & to analyze significant difference between the normal, benign and malignant intestinal mucosa.

3. Materials and Methods

The current study is a prospective ex-vivo HR-MAS NMR analysis of malignant colorectal cancer (CRC) tissue specimens and its corresponding benign tissues.

3.1. Inclusion criteria

All fresh cases of large & small bowel malignancy will be included in our study.

3.2. Exclusion criteria

All the patient who have received neoadjuvant chemo/radiation therapy.

3.3. Chemicals

D2O (99.96 atom% deuterium-enriched, with 0.03% sodium-3-(trimethylsilyl)-2,2,3,3-d4-propionate (TSP)) was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). All other chemicals used were of reagent grade with the highest purity commercially available, unless otherwise mentioned.

3.4. Clinical Methods

Tissue specimens (n=36) were obtained from 18 patients who were enrolled with a written consent to participate in the study that was approved by the ethics committee at King George's Medical University, Lucknow. The mean + SD age of 18 patients who were suffering from colorectal cancer was 50+8.2 years. None of the patients were dehydrated and/or anaemic (mean Hb 11.2g/dL) at the time of surgery. During surgery, two tissue samples weighing 100-200 mg and comprising of tumor and adjacent benign region were obtained from each patient. Sampling was purely based on visual screening by the surgeon. Both tumor and benign region were sampled. All these tissue samples were stored in high-quality plastic vials and snap-frozen in liquid nitrogen

at the time of surgery, to stop all the enzymatic and consequent metabolic activities and were then stored at -80°C till the NMR experiments were performed.

3.5. Tissue Samples

The stored tissues were thawed and washed with D2O prior to NMR analysis, to remove other impurities. The tissues were then dissected carefully and the inner-core (30-40 mg of wet-weight) of the tissues was taken for HR-MAS NMR experiments, to ensure that acquired spectra exhibits resonances from tumor only. Typically, the sample was packed into a 4 mm ZrO2 rotor of 50 μl capacity; a volume of 20 μl of D2O having 0.03% TSP was used as a chemical shift reference. The sample-rotor-setup was then transferred into the HR-MAS NMR probe.

3.6. Magnetic Resonance Spectroscopy

The proton HR-MAS NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer equipped with a 4 mm HR-MAS 1H-13C dual probehead with magic-angle gradient, operating at a proton frequency of 400.13 MHz. In all NMR experiments, the samples were spun at 4.0 kHz in order to eliminate the signals arising due to rotation side-bands. Proton NMR spectra with water suppression were acquired using one-dimensional NOESY pulse-sequence with the following experimental parameters: mixing time τm of 100ms, total relaxation time of 3.99 s, spectral width of 8250.8 Hz, 128 transients with a total recording time of 9 min. Additionally, one-dimensional CPMG (Carr-Purcell-Meiboom-Gill)[14] experiment with water suppression was recorded with an echo-time of 160 ms for each tissue sample. The sample temperature was maintained at 283K using cooled nitrogen gas during the acquisition of the spectra.

The two-dimensional COSY experiment with water suppression was carried out on a few tissue specimens with the following experimental parameters: A spectral width of 4807.69 Hz, 2.2s relaxation delay, 128 transients, and 256 t1 increments were acquired corresponding to a total acquisition time of 7hr. The raw data was zero-filled up to 512w with sine-bell window function prior to double Fourier transformation. Two-dimensional gradient 1H-13C HSQC experiment was performed using adiabatic pulses on few tissue specimens with water suppression. For this experiment, a total relaxation delay of 1.6s was used; 128 transients were averaged for each t1 increment corresponding to a total acquisition time of 8hr. The raw data was zero-filled up to 1024 w and weighted with 90° shifted square-sine-bell window function prior to double Fourier transformation. One-dimensional CPMG spectra with pre-saturation were acquired for few tumor tissues for observing time dependent sample decay, if any. However, no significant differences were observed in the spectral metabolite pattern even after subjecting the tissue specimens for approximately one hour long NMR measurements which is supported by earlier HR-MAS studies on cancer tissue specimens.[15,16] Furthermore, the tissue specimens showing only lipid resonances (comprising of benign region) were re-dissected from the main sample and again subjected to NMR measurements, so as to eliminate the probability of analyzing any wrong section of tissue.

3.7. Histopathology

After spectroscopic analysis, the tissue samples were initially fixed in 10% formalin, and were further embedded using paraffin. 5mm sections at 100 μm intervals were taken to include the whole tissue specimen and were stained using haematoxylin and eosin. Sections were assessed whether or not a tissue specimen proved to be malignant.

3.8. NMR Data Reduction and Pattern Recognition

Both NOESY and CPMG spectra were acquired from tissue samples but only CPMG spectra were subjected for further multivariate analysis as a clear profile of small metabolite biomarkers is obtained due to suppressed resonances from high molecular weight components. Since variations in small metabolite concentrations represent corresponding alterations in intracellular processes, region from 0.56-5.72ppm was considered for Principal Component Analysis (PCA). The spectral complexity of CPMG spectra were reduced by binning the region of 5.72-0.56ppm after removing of the water region (5.02-4.68ppm) to 437 continuous integral segments of equal width of 0.01 ppm by using AMIX software (version 3.7.10, Bruker BioSpin, Switzerland). The data obtained was mean centered, scaled to unit variance and then normalized by dividing each integral segment by total area of the spectrum in order to compensate for the differences in overall metabolite concentration between individual tissue specimens. This resulted in a data matrix, which was then exported to 'The Unscrambler X' Software package (Version 10.0.1, Camo ASA, Norway) for multivariate analysis. PCA was performed on data matrix for the detection of any separation trends using full validation method.

4. Results and Data Analysis

4.1. NMR Spectral Assignments

The HR-MAS proton spectra provided well-resolved resonances for most of the commonly observed metabolites that were assigned on the basis of previously reported values available in the literature.[17–19] The two-dimensional COSY and HSQC experiments were performed on few tissue samples to confirm the assignments of resonance in our spectra (supplementary material, Table 2). The singlet signal at 3.03 ppm, arising due to the methyl group of

creatine, was used as an internal chemical shift reference. Apart from prominently observed lipid resonances, the small metabolites identified include leucine (Leu), isoleucine (Ileu), valine (Val), lysine (Lys), alanine (Ala), lactate (Lac), threonine, acetate (Ace), glutamate (Glu), glutamine, methionine, aspartic acid, citrate, asparagine, tyrosine, phenylalanine, ethanolamine, choline (Cho), phosphocholine (PCho), glycerophosphocholine, myo-inositol, taurine, α- and β-glucose, triglyceride, glycine (Gly) and creatine (Cr).

4.2. Statistical interpretation

The present work has been undertaken with an aim of correlating spectral metabolite profiles with clinicopathological status of patients. Since pathological processes are likely to induce simultaneous changes in an ensemble of metabolites, alteration in the level of a single metabolite may not provide the required insight regarding the biochemical processes occurring at cellular level. Hence, prior to investigating aforesaid correlations, the ensemble of CPMG spectra was subjected to PCA. The 3D PCA score plot having 91% of the explained variance, provided a clear cut group separation (Figure 1) indicating that the metabolic variation in the malignant tissues are distinctively different. The first (PC1) principal component corresponding loading plot has been shown in Figure-2.

Variations occurring in the metabolic profile of malignant and non-malignant tissue specimens could be seen based on clustering of spectra in the PC score-plot. The PCA scores were highly suggestive of group separation. In addition, the altered levels of those metabolites which were responsible for such clustering; in fact, variables contributing to similar information have grouped themselves together in a cluster. The malignant clustering occurs due to increased Val (0.90ppm), Lac (1.34ppm), Ala(1.48ppm) levels of acetate (1.90ppm), glutamate (2.35ppm), taurine (3.23 ppm), choline containing compounds (3.20-3.22ppm), glycine (3.56ppm), lactate (4.12ppm) and α-H of Leu, Ileu, Val, Lys, Ala (3.76-3.79ppm).

The information gathered from clustering in PCA had highly suggested that malignancy induces metabolic perturbations at cellular levels.

5. Discussion

The neoplastic alterations in Kreb's cycle and consequent alterations in normal cellular processes eventually result in malignancy. Humans continue to succumb to cancer, as manifestation of first malignancy increases the risk of recurrence in nearby cells. The initial lesion announces a general susceptibility to adjacent tissues and, therefore, analysis of margins and bed of tumor may define the extent of tumor dispersion, thereby aiding in improved prognosis. If the affected margins are not resected properly, they may

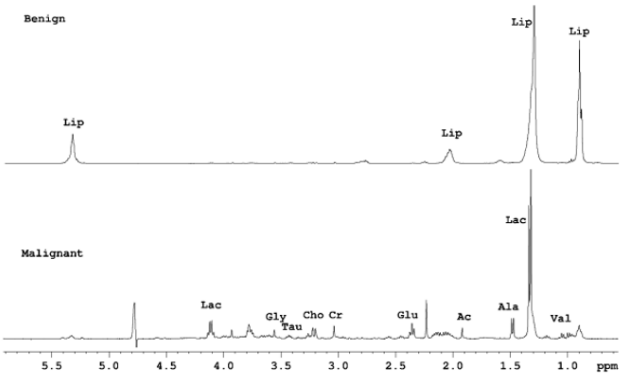

Fig. 1:

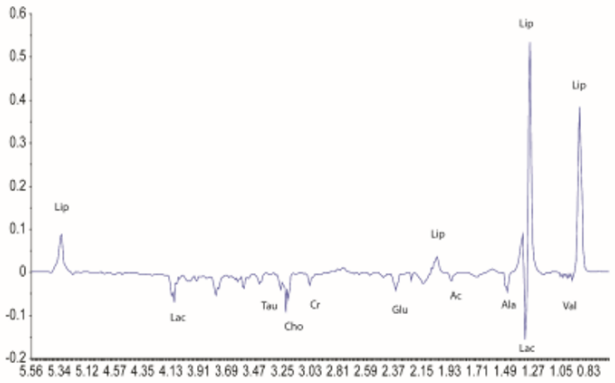

Fig. 2:

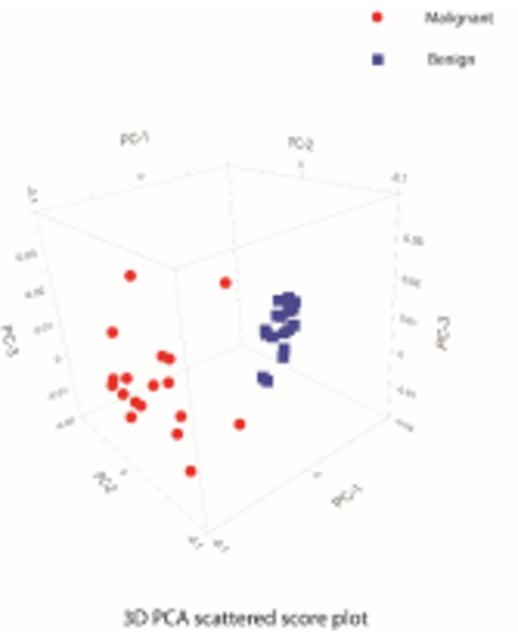

Fig. 3:

result in recurrence and therapeutic failure, making the situation worse. This study aims to define the potential of HR-MAS NMR spectroscopy for a better post-surgical management of CRC patients by defining the malignancy status. The two important findings of this study are: (i) increased levels of Ace, Glu, Lac, Cho, PCho, Gly, Tau, Leu, Ileu, Val, Lys, Ala and decreased levels of fatty acids in malignant tumors as obtained from the loading plots of PCA analysis and (ii) the metabolic alterations of malignant tumors could be distinctly identified from the non-malignant tissues by ^{1}H HR-MAS NMR spectroscopy, which may acknowledge the presence of residual disease in neighboring tissues after resection of the tumor.

The interpretation of PCA model in our case influence of physiological variations.[20] The corresponding loading plot (Figure 2) represented the regions of NMR spectrum that most strongly influence the separation between malignant and control tissue samples. The positive values in loading plot indicated higher concentration of metabolites in benign tissues and a negative value indicates relatively higher concentration with respect to oral SCC tissue specimens. The regression coefficients indicated that acetate (1.90ppm), glutamate (2.35ppm), taurine (3.23 ppm), choline containing compounds (3.20-3.25ppm), glycine (3.56ppm), lactate (4.12ppm) and α-H of Leu, Ileu, Val, Lys, Ala (3.76-3.79ppm) were present in higher concentration in malignant.

In our study, there is an observed decrease in lipid concentration in cancer cells which may be due to the modifications in lipid composition under malignant conditions. These modifications include decrease in lipids which consequently cause alterations in membrane bilayer fluidity and enzymatic activities at the level of mitochondrial and microsomal membranes, which has been proved by an earlier biochemical study on rapidly growing hepatoma cells[21] Malignant cells exhibit uncontrolled rates of cell division and this process requires lipids, amino-acids and other small metabolites to support key biosynthetic pathways. Variations in these metabolite concentrations reflect changes in metabolism arising from biologic condition of disease. Thereby, in this study emphasis had been laid upon small metabolites which provide a true picture of cell proliferation and the altered cellular processes. The higher levels of Lac, PCho, Cho, amino acids and lower levels of lipids were found only in typical malignancy as compared to non-malignant tissue specimens; this is in concordance with the metabolic alterations occurring in malignant cells during cell–proliferation, as it requires nutrients, energy and higher biosynthetic activity to duplicate all macromolecular components during each passage through cell-cycle while maintaining the cancer cell homeostasis. Therefore, cellular processes occurring in malignant cells are quite different from those of non-proliferating cells. The higher lactate levels have been observed in our study (Figure 1) as Otto Warburg[22] reported that metabolic activities of malignant cells show high glycolytic rates and reduced mitochondrial oxidation, which favors the cell-survival in hypoxic micro-environment of tumor; also, glucose to lactate conversion protects malignant cells from oxidative stress (due to reactive oxygen species) resulting in high lactate accumulation as a pronounced consequence.[23] Apart from lactate, higher levels of alanine are also attributed to the hypoxic conditions of malignant tumors.[24]

The rapid proliferation of cancer cells also affects their energy requirement which are fulfilled by high-energy creatine phosphate, which is formed from creatine by the mediation of enzyme creatine kinase.[25] The formation of high energy phosphates result in creatine deficit in the cells which has been reinstated by our findings. In the present investigation using HR-MAS, broad resonances due to choline-containing compounds are well–resolved in the CPMG spectrum, providing precise information about the metabolic perturbations occurring in cancer cells. Choline (3.20 ppm) is known to play a key role in phospholipid metabolism of cell membranes, and represent active cell proliferation in the form of increased membrane phospholipid turnover. The phosphorylation of choline results in PCho, which is followed by a cascade of biochemical reactions to form phosphatidylcholine. Phosphatidylcholine is a major phospholipid component of eukaryotic cells which participates in lipid metabolism, signal transduction mechanisms and in membrane structure.[25] Thus, higher Cho and PCho contents are well-known markers of increased cell proliferation in the cancer cells. Apart from Cho and PCho, higher level of acetate was also observed which may be attributed to the increased lipid biosynthesis and altogether these three metabolites reflect highly active proliferation of tumor cells.

The increased glutamate levels in tissues reconfirm a recent study on tumor growth which has shown that glutamate stimulates proliferation and migration of tumor cells and thus facilitates the tumor progression.[26] Glycine is an amino acid which is reportedly an essential precursor for de novo purine formation (an integral part of nucleic acids) as shown by an earlier study on breast tumor cell lines.[24] Hence, its higher concentration in malignant tumors is a marker for enhanced nucleotide synthesis.

The unique feature of cancer as a disease is the capacity of the disease itself to use amino acids for its own metabolism and proliferative activities. The cancer cells utilize glutamine, alanine and glycine along with other branched chain amino acids for its protein biosynthesis,[27] which increases manifold in malignant tissues for their proper progression and proliferation.[28] This fact is reflected by the significant increase in the relative intensities of these metabolites in NMR spectra of the malignant tissues.

Not only enhanced protein synthesis, lipidogenesis and nucleic acid formation contribute in unhindered cancer proliferation but volume regulation and osmoregulation also play an equally important role in it. Taurine acts as an osmolyte[24] and its higher concentration probably helps in controlling hypoxia induced cell swelling,[29,30] thereby maintain the cell volume. Taurine also acts as a free-radical scavenger for reducing the oxidative stress of the cells and their unhindered proliferation.[31] Earlier studies on various cancers have shown that taurine concentration is higher in various tumors. All these metabolites act as biomarkers for the suppression or aggravation of different cellular processes occurring in cancer cells and therefore, play an important role in distinguishing normal tissues from the malignant ones.

The limitation of this study may be attributed to the smaller sample size, therefore, large population studies are required to ensure the role of these biomarkers in cancer cells and the study can be further extended to understand the aggressiveness of the tumor including metastasis.

Since tissue extraction methods may modify original cellular metabolic composition to an unknown extent, particularly the proportion of acetate and choline derivatives,[32] these procedures may cause erroneous correlation between diseased state and MRS results. Furthermore, tumor heterogeneity limits the utility of extraction processes[32] while in in-vivo studies, peaks being broad in nature may mask the signals arising from neighboring protons. HR-MAS spectroscopy has been employed to overcome such modifications in metabolic profile of tissue samples, and the spectral resolution is quite comparable to that of solution state NMR. The overall representation of metabolic alterations in this prospective 1H HR-MAS NMR study goes hand-in-hand with the tumor biology, which is explained by the results of multivariate statistical analyses. In other words, the metabolic fingerprints are easily identifiable.

The results obtained in the present study may contribute to the evaluation of tumor biochemistry and its point-to-point correlation with histopathology suggests its clinical relevance and it may further augment our understanding of cancer cell metabolism at the level of metabolic activity.

Since, the spectral measurements were directly conducted with intact tissue specimens, therefore, results obtained are closely related to the in-vivo status of tumors. Ex-vivo and in-vivo NMR spectral peak observations in pathological states can support the reliability of clinical applications by providing more precise information about the biochemical status of the disease. Earlier studies on brain tumors reinforce this fact by determining the relationship between in-vivo and ex-vivo biochemical characterization of brain tumors by using MR spectroscopy.[16,33] Analysis on large sample size is therefore required in order to prove our findings.

6. Conflict of Interest

There is no potential conflict of interests related to the exclusive nature of this paper.

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