Original Research Article

The role of in-vivo proton magnetic resonance spectroscopy in gallbladder cancer patients: A metabolomic approach

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Received: 26-11-2021 / Revised: 21-12-2021 / Accepted: 08-01-2022

Abstract

Background: Gallbladder carcinoma (GBC) is considered as a rare disease but it is the fifth most common malignancy of the gastrointestinal tract. Many diagnostic test are used to diagnose a case of GBC comprises laboratory studies and imaging tests.Proton MR Spectroscopy is able to detect and identify a large number of low concentration metabolites. It helps in detailed understanding of metabolic basis of physiology of disease and to diagnose the disease in its early stage.In this study we aim to identify the metabolides in benign malignant and normal gall bladder tissue and compare the metabolic profile of gall bladder tissue with histopathology report. **Methods:** The cross sectional study, total 119 patients were included in this study on the basis of well explained inclusion and exclusion criteria. Group I includes 51 patients with carcinoma gall bladder, Group II consists of 41 patients of benign gall bladder disease and Group III contain 27 patients with diseases other than hepato-biliary disease. ^{1}H NMR metabolic profiling was done for the detection in metabolic changes. The Chi-square test, student's t test and ANOVA were used for statistical analysis. **Results:** The area under curve of choline peak was significantly greater in group I (66.71) as compared to group II (14.85) and group III (5.44). The Lipid AUC and Lipid/Choline Ratio were significantly reduced in group I (222.61 and 5.01) as compared to group II (242.46 and 22.81) and group III (254.56 and 71.32). The Bilirubin total, Bilirubin Direct, SGPT, SGOT and ALP were significantly higher in group I. The change of MRI mass/ stone size was significantly positive correlated with choline AUC. **Conclusion:** The in-vivo MRS is a good tool to differentiate the malignant and benign Gallbladder tissue on the basis of metabolic profile of Gallbladder tissue with short time consuming, with no increased risk of contamination and metastasis of disease and good tool to differentiate between malignant and benign.

Keywords: MR Spectroscopy, Carcinoma gallbladder, Chronic cholecystitis, Gallstone disease, Metabolomics

Introduction

Gallbladder carcinoma (GBC) is considered as a rare disease but it is the fifth most common malignancy of the gastrointestinal tract. The gall bladder incidence varies by racial group and worldwide approximately 2.5 per 100000 persons[1,2]. Gall bladder cancers is rare in India with incidence rates of 0.5 and 1.3 per 100 000 population in men and women, respectively GBC cases are predominantly reported in North and Northeast India along the Ganga and Brahmaputra region[3,4]. Most of the cases of gallbladder cancers are unidentified until a person goes to a doctor when have symptoms like pain in abdomen, jaundice, and lump in upper abdomen.

Many diagnostic test are used to diagnose a case of GBC comprises laboratory studies and imaging tests. Laboratory studies include liver function tests, CA 19-9 assay, Carcinoembryonic antigen (CEA) assay[5,6]. Ultrasound is the first imaging test done in people presented with jaundice or pain in the right upper part of abdomen that might be caused by gallbladder problems.Computed tomography scan are often used to help diagnose gallbladder cancer by showing tumors in the area, staging the cancer. This can help determine operability and guide a biopsy needle into a suspected tumor or metastasis. For this procedure, called a CT-guided needle biopsy[7].

Biopsy of a suspicious area in the gallbladder is the best way to confirm if it is cancer. But a biopsy may not always be done before surgery. Doctors are often concerned that during biopsy from tumor, cancer cells can spread to other areas[8,9].

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Proton MR Spectroscopy is a specialized analytical technique associated with magnetic resonance imaging (MRI)[10,11]. It is a non-invasive, ionizing-radiation-free analytical technique that has been use to study metabolic changes in CNS disorders and other disorders of muscles. Both techniques use signals from hydrogen protons (1H) MRS allows doctors and researchers to obtain biochemical information about the tissues of the human body in a non-invasive way, without the need for a biopsy, whereas MRI only gives them information about the structure of the body. The application of the non-invasive methodology of ^{1}H-MR spectroscopy is to the qualitative and quantitative assessment of human gall bladder tissue *in vivo*. MRS can detect subtle changes in biochemistry because of high resolution following the metabolic alteration in the early stages of disease in single experiment[8,9]. NMR spectroscopy is quantitative and does not require extra steps for sample preparation, such as separation or derivatization.

Metabolomics also known as Metabolic profiling is the qualitative and quantitative analysis of numerous low molecular weight metabolites (typically <1000 Da) present in cells, biofluids, pathological fluids, tissue, tissue extracts such as organic acid, carbohydrates, alcohol, amino acid, phospholipids without separation of individual components[12]. Majority of the metabolites in biological samples are present in very low concentration. Proton MR Spectroscopy is able to detect and identify a large number of low concentration metabolites. It helps in detailed understanding of metabolic basis of physiology of disease and to diagnose the disease in its early stage [12-14]. It is used for early diagnosis, prognosis and monitoring therapy, identification of new diagnostic and therapeutic targets, to know altered metabolic profile in inborn error of metabolism eg. Homocystinuria, phenylketonuria.In this study we aim toidentify the metabolides in benign malignant and normal gall

bladder tissue and compare the metabolic profile of gall bladder tissue with histopathology report.

Materials and methods

The cross sectional study was conducted at the Department of General Surgery, King George's Medical University, Lucknow during September 2017 to August 2018. Ethical clearance has been obtained from the institutional ethics committee. Total 119 patients were included in this study on the basis of well explained inclusion and exclusion criteria. All new cases of Ca GB diagnosed by clinically and Radiologically, Benign gall bladder disease and healthy voluntary with 20-65 years old individual were included in this study. Patients with previously treated cases of carcinoma gallbladder cancer and metal plate, pin, or other metallic implant e.g.; intrauterine device, such as Copper-7 IUD; insulin or other drug pump; aneurysm clips; previous gunshot wound; cochlear implant or other hearing device; permanent (tattoo); cardiac pacemaker or artificial heart valve were excluded from the study. Written an informed consent is taken from all patients included in study.

Group I includes 51 patients with carcinoma gall bladder, Group II consists of 41 patients of benign gall bladder disease and Group III contain 27 patients with diseases other than hepato-biliary disease.

Detailed history and examination, blood investigation including CBC, serum electrolytes, KFT, LFT, RBS, PT/INR, HIV, HCV, HBsAg , USG Whole abdomen were recorded.

MRS data were acquired from patients with USG whole abdomen finding suspicion of Carcinoma Gallbladder, benign gallbladder disease and patients admitted to our department with diseases unrelated to Gallbladder.

After optimizing the scanning parameters MRS data acquired from 125 subjects. Out of them 6 were excluded due to motion artifact due to failed respiratory gating and due to very noisy spectrum. The subjects fasted for at least 6-8 hours before MR spectroscopy. The MR experiments were performed on GE medical systems, Signa Excite Gemsow 1.5 T. The GB was localized using a T2 weighted image and in last 10-30% of quite expiration respiratory-gated PRESS (point resolved spectroscopy) sequence with Echo time (TE) 144ms and repetition time (TR) 1500ms was used after automatically shimming the volume of the right upper quadrant to between 6Hz to 25Hz. The shortest Echo time available to the sequence was chosen to maintain the signal from short T2 metabolites. A total 144 individual signal acquisition were acquired in each study. A 20 X 20 X 20 mm3 voxel was positioned in the gallbladder using these images.(Figure 1,2)

Figure 1 [A,B,C,D]: The MR Spectrum of Malignant Gall bladder tissue, peak of phosphatidylcholine and lipid, position of voxel

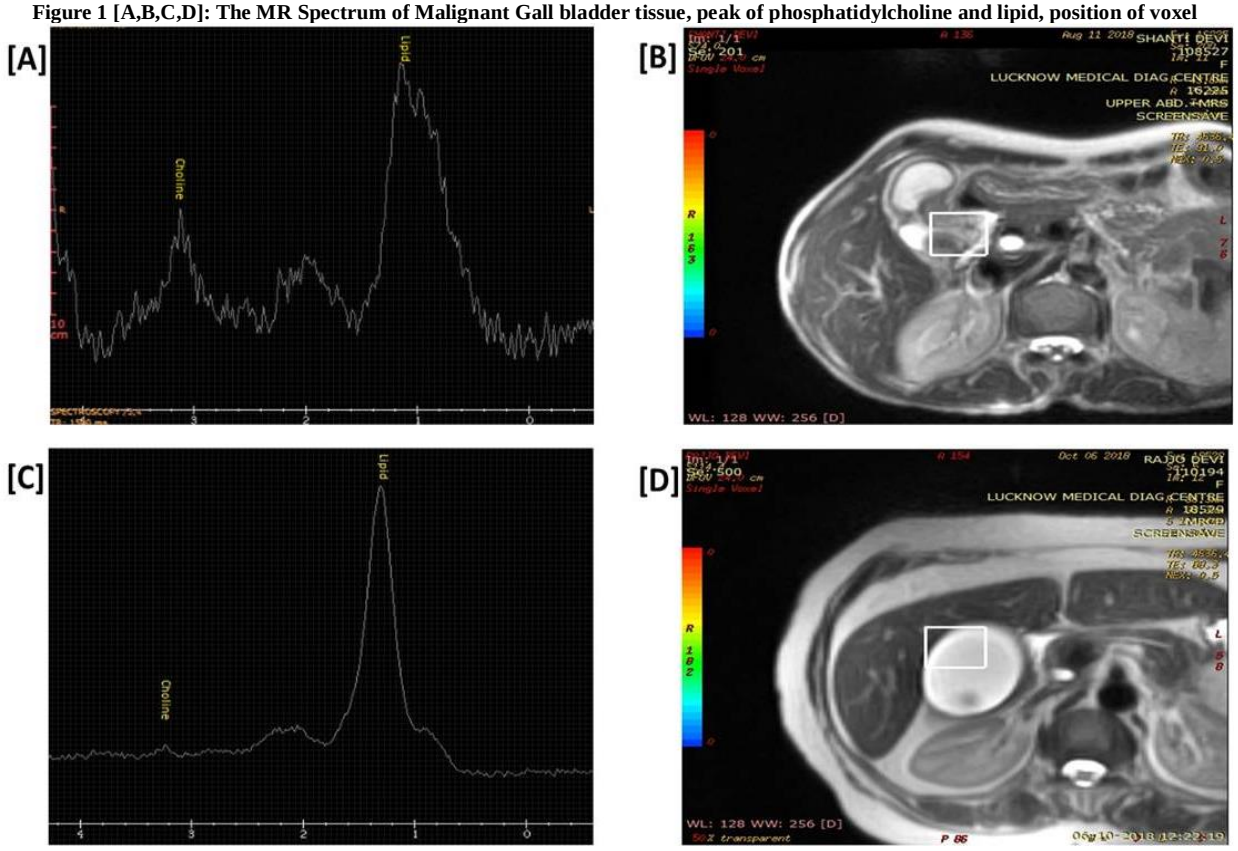
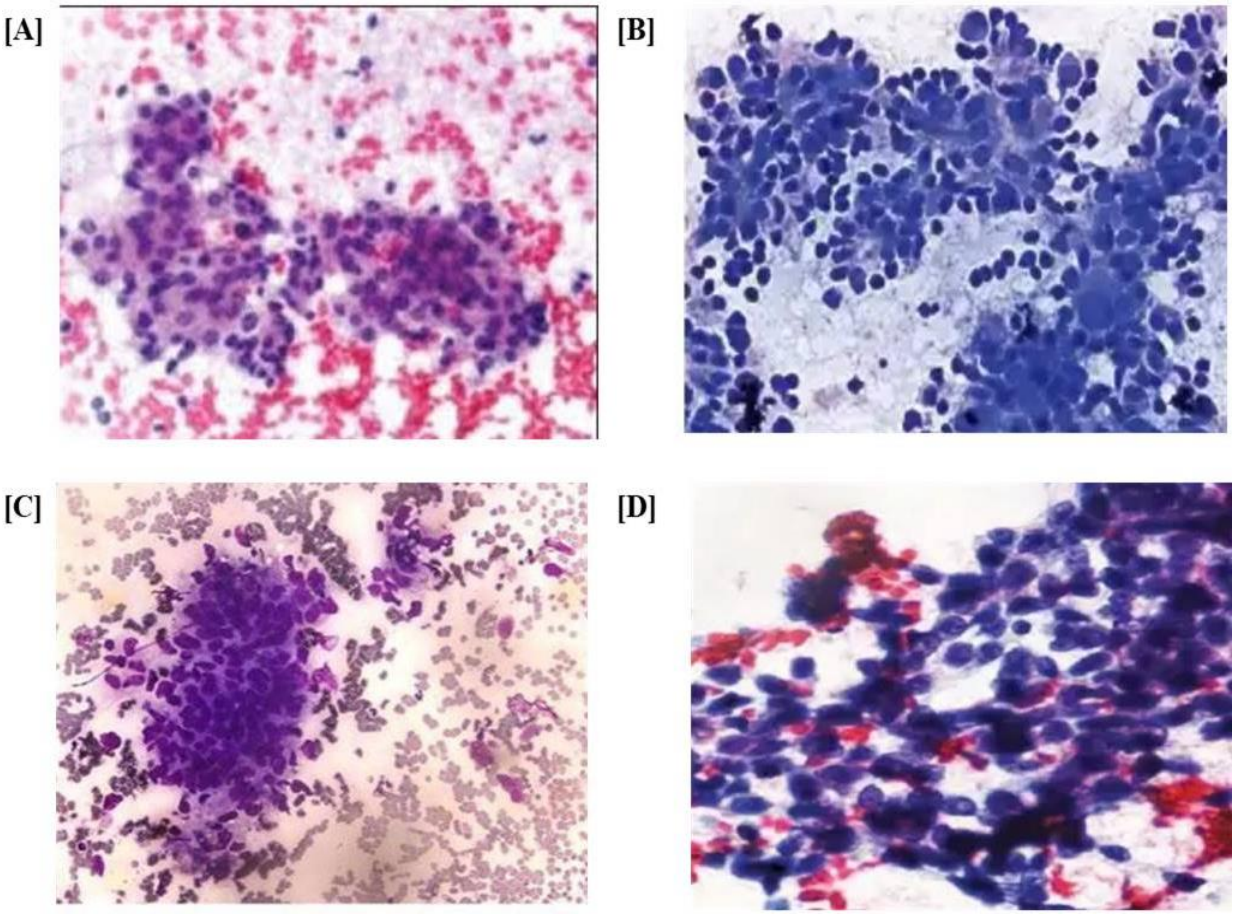


Figure 2 [A,B,C,D]: The MR Spectrum of Normal Gall bladder tissue, peak of lipid ,and there is no peak of Phosphatidylcholine, position of voxel



The assignment of peaks was based on results from previous in vitro studies. The peak at 3.20 ppm was assigned group of Phosphatidylcholine (PhC) and 1.3 ppm was for lipid peak.

The quantitative analysis of metabolites done by plotting gridlines on spectrum and measuring the area under curve(AUC) i.e. number of squares in arbitrary unit (a.u.). The area under curve of the each metabolite's peak on spectrum will refer to the concentration of that compound in target tissue.

To conduct the study the sample was collected by surgical resection of Gallbladder (radical cholecystectomy) in operable cases and Laparoscopic/ open cholecystectomy in cases of benign Gallbladder disease from Department of General surgery, Department of Surgical gastroenterology and Department of Surgical Oncology, KGMU, Lucknow, which was kept in 10% formalin preservative and by USG guided FNAC in inoperable cases of carcinoma Gallbladder from department of Interventional Radiology, KGMU, Lucknow, slides made and fixed in spirit.

The tissue was labelled and subjected to Histopathological processing in Department of Pathology, KGMU, Lucknow. From each sample 4-6 slides were prepared. Slides was stained by Hematoxylin and eosin stain. (Figure 3 and 4)

Figure 3: Hematoxylin and eosin stained slide showing Adenocarcinoma Gallbladder showing marked nuclear pleomorphism and frequent mitosis

Figure 4: Hematoxylin and eosin stained slide showing Chronic Cholecystitis ,marked inflammatory cells present.

Statistical Analysis

The Chi-square test was used to compare the dichotomous/categorical variables. The unpaired Student's *t* test was used to compare discrete variables. ANOVA was used for comparing the more than three groups. A p-value less than 0.05 were considered significant. SPSS 21.0 software was used for statistical analysis.

Results

Table 1 show the comparisons of age and sex in between groups I, II and III. The maximum incidence of carcinoma gall bladder is found in 36 to 50 year age group i.e. 62.7% (32 out of 51) and the maximum incidence of benign Gall bladder disease and other than hepato-biliary disease were found in <50 year age group. The frequencies of distribution of patients according to different age group were significantly associated with groups. The sex ratio in our study population showed that female patient proportion was higher than males in group I (74.5% and 25.5%) and group II (78.0% and 22.0%, respectively). The distribution of gender among three group were not significant (p-Value= 0.373).

Table 1: Comparisons of age and sex in between groups I, II and III

		Group I (n=51)		Group II (n=41)		Group III (n=27)		p-Value
		n	%	n	%	n	%	
Age (years)	25 to 35 years	2	3.9%	16	39.0%	13	48.1%	<0.001
	36 to 50 years	32	62.7%	13	31.7%	7	25.9%	

	51 to 60 years	10	19.6%	11	26.8%	6	22.2%	
	Above 60 years	7	13.7%	1	2.4%	1	3.7%	
Sex	Male	13	25.5%	9	22.0%	10	37.0%	0.373
	Female	38	74.5%	32	78.0%	17	63.0%	

Table 2 shows the comparisons of USG stone, MRI (Liver Sol), choline peak, and MRS lipid/lactate peak in between groups I, II and III. The USG was depicting the presence of gall stone in different groups. The gall stone was significantly more 33 (64.7%) in group I, 41 (100%) in group II, as compared to group III (0%). The presence of space occupying lesion was significantly high in group I (Carcinoma gall bladder) patient (41.2%) as compared to group II (0%) and group III (0%). The presence of choline peak is significantly high in Group I (82.4%) as compared to group II (53.7%) and group III 14.8%). The Lipid/Lactate peak in MR spectroscopy was no significantly different in between group I, group II and group III (p = 0.511).

Table 2: Comparisons of USG stone, MRI (Liver Sol), choline peak, and MRS lipid/lactate peakin between groups I, II and III

		Group I		Group II		Group III		p-Value
		n	%	n	%	n	%	
USG Stone	Present	33	64.7%	41	100.0%	0	0.0%	<0.001*
	Absent	18	35.3%	0	0.0%	27	100.0%	
MRI (Liver Sol)	Present	21	41.2%	0	0.0%	0	0.0%	<0.001*
	Absent	30	58.8%	41	100.0%	27	100.0%	
Choline Peak	Present	42	82.4%	22	53.7%	4	14.8%	<0.001*
	Absent	9	17.6%	19	46.3%	23	85.2%	
MRS Lipid/ Lactate Peak	Present	50	98.0%	41	100.0%	27	100.0%	0.511
	Absent	1	2.0%	0	0.0%	0	0.0%	

*=Significant (p<0.05)

Table 3 shows the comparisons of mean Mass/stone size in between group I and group II. The mean size of mass/stone was significantly higher in group I (35.75 mm) as compared to group II (12.41mm).

Table 3: Comparisons of MRI MASS/ STONE size in between group I and group II

	Group	N	Mean	Std. Deviation	p-value
MRI MASS/ STONE size	Group I	51	35.75	23.90	<0.001*
	Group II	41	12.41	4.76	

*=Significant (p<0.05)

Table 4 shows the area under curve of choline peak and Lipid peak in arbitrary unit on MR spectroscopy along with the ratio of lipid to choline. The area under curve of choline peak was significantly greater in group I (66.71) as compared to group II (14.85) and group III (5.44). The Lipid AUC and Lipid/Choline Ratio were significantly reduced in group I (222.61 and 5.01) as compared to group II (242.46 and 22.81) and group III (254.56 and 71.32). The inter-group comparison (Tukey post-hoc test), the choline AUC was significantly different in between group I vs group II and group I vs group III, Lipid AUC was significantly different in between group I vs group II only. Lipid/Choline Ratio was significantly different in between group I vs group II; group I vs group III and group II vs group III.

Table 4: Area under curve of Choline peak and Lipid peak in arbitrary unit on MR spectroscopy along with the ratio of lipid to choline

		N	Mean	Std. Deviation	p-value
Choline AUC (in a.u.)	Group I	51	66.71	45.27	<0.001*
	Group II	41	14.85	10.60	
	Group III	27	5.44	5.27	
Lipid AUC (in a.u.)	Group I	51	222.61	52.80	0.016*
	Group II	41	242.46	51.79	
	Group III	27	254.56	30.19	
Lipid/Choline Ratio	Group I	51	5.01	3.54	<0.001*
	Group II	41	22.81	14.64	
	Group III	27	71.32	34.84	

*Significant (p<0.05).

Table 5: The value of Liver function test(LFT) that include total bilirubin, direct bilirubin, SGPT, SGOT and ALP and LFT which is Group I > Group II > Group III. The Bilirubin total, Bilirubin Direct, SGPT, SGOT and ALP were significantly higher in group I (6.50, 4.46, 79.05, 95.88 and 760.30) as compared to group II (2.09, 1.44, 62.96, 80.48 and 500.27) and group III (1.05, 0.58, 37.01, 42.79 and 151.63). The inter-group comparison (Tukey post-hoc test), the bilirubin total and bilirubin direct were significantly different in between group I vs group II and group I vs group III. Whereas the SGOT and ALP were significantly different in between group I vs group III only.

Table 5: Comparisons of Liver function test (LFT) in between Group I, Group II and Group III

		N	Mean	Std. Deviation	p-value
Bilirubin Total	Group I	51	6.50	7.33	<0.001*
	Group II	41	2.09	1.75	
	Group III	27	1.05	0.58	
Bilirubin Direct	Group I	51	4.46	5.10	<0.001*
	Group II	41	1.44	1.21	
	Group III	27	0.58	0.35	
SGPT	Group I	51	79.05	90.52	0.066
	Group II	41	62.96	76.94	

	Group III	27	37.01	15.06	
SGOT	Group I	51	95.88	99.04	0.025*
	Group II	40	80.48	81.75	
	Group III	27	42.79	13.91	
ALP	Group I	51	760.30	603.90	0.001*
	Group II	41	500.27	913.02	
	Group III	27	151.63	81.55	

Correlation of the MRI MASS/ STONE size with CHOLINE AUC (in a.u.) is shown in Table 6.The change of MRI MASS/ STONE size was significantly positive correlated with CHOLINE AUC (in a.u.).

Table 6: Correlation of the MRI MASS/ STONE size with CHOLINE AUC

	Pearson Correlation	p-Value
MRI MASS/ STONE size with CHOLINE AUC (in a.u.)	0.341**	0.001

Discussion

In recent years, HR-MAS NMR spectroscopy has been successfully applied to analyze the metabolic composition of control and pathological tissues from brain (Wright et al. 2010), pancreas, lung, breast, colorectal cancer[15-19]. Therefore in the present study, ^{1}H Magnetic resonance spectroscopy based metabolomic approach has been applied with an aim to carry out metabolic profiling of gall bladder tissues followed by Histopathological analysis of the Gall bladder pathology, for confirmation of benign and malignant tissues types.

In-vivo MR spectroscopy has been used to analyze the level of metabolites in patient of Hepatobiliary diseases for which 125 patients were included out of them 6 were excluded due to motion artifact, failed respiratory gaiting and very noisy spectrum on spectroscopy. Total 119 patients were included in the study of which group I includes 51 patient are of carcinoma gall bladder, Group II consists of 41 patients of benign gall bladder disease and Group III contain 27 patients with diseases other than hepato-biliary disease. The study samples are of between 25-65 years of age with mean age of 45.19±11.51 years.

The various previous studies reported that the Carcinoma gallbladder is more common in females[1], such mutagenic toxins secreted reside more longer due to stasis because of impaired contractility associated with the female hormone, progesterone. This prolonged exposure allows environmental carcinogens to then cause malignant transformation, helping to reconcile the theory of 'seed versus soil' and incorporate the predilection to the development of gallstones (also requiring some gallbladder stasis)[2] and in our study we have found that out of 51 GBC patient,13 (25.5%) are male and 38(74.5%) are female i.e. GBC is almost 3 times more common in females. These results were consistent with the results of other studies[20-23], where it was reported to be 1:3, 1:3,1:2.5 and 1:2-3 respectively.

Recent studies commented on the evidence that the incidence of GBC is increasing in younger age group. In our study 66.6% of GBC patient are below 50 years of age group. Majority of cases are within 36-50 years of age group (62.7%), which is considering highly significant. The Gall stone that has been linked as a well-known risk factor for GBC, as gallstones causes chronic inflammation of gallbladder and chronic inflammation can cause cellular atypia. In our study it was found that gall stone was present in 64.7 % of GBC cases that is highly significant with p-value, which is comparable to a study from MD Anderson Hospital[24] in which 51 (88%) patients had gallstones. Other study also reported that the presence of gall stones in 70% and 90% respectively in gallbladder cancer patients[22,25].

In a study from United States shows that the increasing stone size (>3 cm) are associated with increasing risk of GBC[26]. Persons with stones larger than 3 cm have ten times higher risk of developing GBC as compared to those with stones smaller than 1 cm.10 GB packed with stones (high stone / GB volume ratio) are more likely to have GBC. In our study among the patient of GBC with Gallstone, 60% patient of GBC have Gall stone size >3 cm. which is similar to the results of the studies by Lowenfels et al., 1989[27] and Diehl, 1983[28].

Most of the patient of GBC presents in advance stages, the lack of serosal layer of gallbladder adjacent to the liver thus enabling hepatic invasion and metastatic progression is one of the major cause of its miserable prognosis. In our study we have found that 41% of GBC patient was detected having Hepatic involvement as hepatic infiltration or space occupying lesion in liver suggestive of liver metastasis on MRI.

The adenocarcinoma is a cancer that starts in gland-like cells that line many surfaces of body, including digestive system. Adenocarcinoma is the most common histological subtype of GBC approximately 90-95% of all cases, in our study we found that all the GBC patient have adenocarcinoma subtype on histopathology report.

The amount of choline (i.e. Area under curve) on MR spectroscopy was found significant higher in GBC patient when compared with patient of gall stone disease and normal individuals. Choline is a precursor of acetylcholine, component of cell membranes. Choline is a marker of cellular membrane turnover and therefore elevated in neoplasm, inflammation. Nagana Gowda et al. (2009) also found elevated choline-containing metabolites in tissue specimen of Gallbladder carcinoma[29].

In our study The lipid profile of gall bladder tissues followed Normal GB> Benign GB > GBC Gallbladder tissue trends as observed by MR spectroscopy, which supports earlier study on lipids extract of gall bladder tissues[30]. Lipids are the main fuel source in mammalian cells for new cell production. Cancerous cells have rapid proliferation and growth of cells where energy consumption is more than energy production causes utilization of stored lipids and ultimately lipid depletion in cancer cells[31].

The patient with benign gallbladder disease was also show significant higher level of Choline on spectroscopy when compared with the normal individual. These significantly increased value possibly suggestive of role in pathogenesis of disease.

In our study we found that the amount of choline in MR spectroscopy is higher in patients with larger the mass or stone size on MRI. There was significant correlation at the level of the 0.01 level (2-tailed), possibly suggestive of role in pathogenesis of disease.

Conclusion

The in-vivo Magnetic Resonance spectroscopy is an adequate option for evaluating the metabolic profile of Gallbladder tissue without extraction of tissue, sample preparation and acquisition, which is time consuming, laborious and increase the chance of infection and risk of metastasis in case of malignant disease. We found that the in-vivo MRS is a good tool to differentiate the malignant and benign Gallbladder tissue on the basis of metabolic profile of Gallbladder tissue. As the technique is less time consuming, with no increased risk of contamination and metastasis of disease and good tool to differentiate between malignant and benign Gallbladder disease, making the technique more useful for clinics.

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Conflict of Interest: Nil Source of support: Nil