Original Article

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Surgery Section

Expression of CCK Receptors in Carcinoma Gallbladder and Cholelithiasis: A Pilot Study

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ABSTRACT

Background: Gastrin and cholecystokinin (CCK) receptors are trophic for various gastrointestinal malignancies. Their role in gallbladder cancer has not been widely studied.

Objectives: To identify expression of CCK-A and CCK-B receptors in the tissue and blood of patients suffering from carcinoma (CA) gallbladder and gallstone disease and to compare expression of CCK A and B receptors in the gall bladder tissue and blood of healthy individuals and patients of CA gallbladder, and gallstone diseases.

Materials and Methods: Forty nine subjects of both genders were recruited, comprising of 22 patients of CA gall bladder, 19 cases of cholelithiasis and, 8 normal gallbladders obtained from patients operated for trauma of the biliary system or Whipple's procedure. RNA extraction and cDNA formation for CCK-A and CCK-B receptors were carried out. Real Time PCR was performed on cDNA and threshold cycle (Ct) value of each sample was obtained and ΔCt was calculated. Chi-square test for comparing two groups and ANOVA test for comparing multiple groups were applied and if $p<0.05$ then Dunnett-C test was performed.

Observation and Results: Both CCK-A and CCK-B receptors were expressed irrespective of its origin in all tissues and blood samples studied; be it normal, Cholelithiasis or CA gallbladder and there was no difference among them ($p>0.05$).

Conclusion: This preliminary study showed higher expression of CCK-A receptors in patients of cholelithiasis and decreased expression of CCK-A receptors in patients of CA gallbladder as compared to normal gallbladder although it did not rise to statistical significance.

Keywords: Cholecystokinin receptor, Gastrointestinal malignancies

INTRODUCTION

Carcinoma (CA) of the Gallbladder commonly occurs in elderly patients; most tumours are diagnosed at advanced stage and carry poor prognosis [1]. It is a great diagnostic and therapeutic challenge for surgeons as 5 year survival of patients in most series is < 5% and median survival is < 6 months [1,2].

The incidence of CA Gallbladder varies widely in different geographic regions, and racial and ethnic groups. The incidence is highest in Chile, North-east Europe, Israeli Jews, and Americans of Indian and Mexican origin [3]. In India, it is the most common form of biliary malignancy and third most common CA of the digestive tract in Eastern UP and Western Bihar [4] and fifth most common gastrointestinal CA in women [5-7].

It is well established that gastrin and cholecystokinin (CCK) are trophic for various gastrointestinal malignancies [8-10]. It is also known that gallbladder has high concentration of Cholecystokinin-A (CCK-A) receptors [11]. CCK-A receptor does not modulate the susceptibility of cancer gallbladder [12]. But their role in gallbladder malignancy remains undecided. Molecular studies in high incidence areas, and in subsets of high risk gallbladder disease patients, may help to predict the possibility of gall stone disease developing into cancer and may dictate for measures to be taken in developing new screening or therapeutic strategies.

"The aim of this study was to find out expression of CCK receptors in two common gallbladder diseases in adults". The objectives were as follows.

OBJECTIVES

1. To identify expression of cholecystokinin receptors, CCK-A and CCK-B, in the gallbladder tissue and blood of patients suffering from CA gallbladder and cholelithiasis.
2. To compare expression of these receptors in above disease states in similar samples of healthy subjects.

MATERIALS AND METHODS

This prospective study was conducted in the Department of General Surgery, Era's Lucknow Medical College and Hospital, Lucknow, India from March 2008 to December 2009, after obtaining approval from the Ethical Committee of the institution. Patients' samples were also taken from Chhatrapati Shahuji Maharaj Medical University (CSMMU), Lucknow, India. The analysis of CCK –A and CCK-B receptors was done in the Indian Institute of Toxicology Research (IITR), Lucknow, India.

In all, 49 cases were recruited, after obtaining informed consent. They included 22 patients of cancer gall bladder, 19 cases of cholecystitis with cholelithiasis and eight normal gallbladders; which were obtained from patients operated for trauma of the biliary system or Whipple's procedure, and served as control. Demographic data of all the cases were maintained. Gall bladder tissue and blood samples were taken from all patients and control subjects.

Ultrasound (USG) abdomen of all patients was done prior to surgery. CT scan abdomen was carried out in only those patients in whom suspicion of CA gallbladder was present on ultrasound examination of the abdomen.

CCK-A and CCK-B receptors analysis: Gallbladder tissue samples were obtained and immediately submerged in Trizol solution for storage without jeopardizing the quality or quantity of RNA. Tissue pieces were taken in 600 μl of Trizol solution. The volume of tissue was 1/10th of Trizol solution and stored at -80°C for analysis of CCK receptors. Rest of the gallbladder tissue was sent for histopathological examination.

Two ml of venous blood was taken from all above patients and control subjects in an EDTA vial and immediately 100 μl of EDTA blood was put in 600 μl of Trizol solution and stored at -80° C for analysis of CCK receptors. In advanced inoperable cases of CA gallbladder only blood samples were taken from the patients.

Total RNA was extracted to study the expression of CCK-A and CCK-B receptors by RT-PCR (Reverse Transcriptase- PCR) and the quantization was done by Real Time PCR using SYBR green based chemistry.

METHOD OF RNA EXTRACTION

Samples collected and stored in Trizol solutions at -80°C were homogenized. Transferred 200 µl of homogenized sample to a vial containing 600 µl of Trizol, and then proceeded as follows:

1. Homogenized once more.
2. 120 µl of chloroform was added in each vial.
3. Vortex for 15 seconds.
4. Incubated at room temperature for 15 minutes.
5. Centrifuged the mixture at 10,000 Xg for 15 minutes. The mixture was separated into 3 phases. The colourless upper aqueous phase (containing the RNA), the semisolid inter phase (containing most of the DNA) and the lower organic phase.
6. Without disturbing the inter phase, carefully transferred the aqueous phase, into tube containing 500 µl of Isopropanol, mixed gently and incubated at room temperature for 10 minutes.
7. Centrifuged at 10,000 Xg for 15 minutes. Pellet was visualized at the base containing RNA precipitate and the supernatant was discarded.
8. Wash the pellet with 1 ml of 80% ethanol and supernatant was discarded.
9. Air-dried the pellet for about 30-60 min: at room temperature.
10. Pellet was dissolved in 100 µl of Diethylpyrocarbonate (DEPC) water. (RNAse free)
11. Quantification of RNA was done with the help of Pico drop.

The RNA which was extracted in pellet form was used for cDNA formation.

RT–PCR of CCK-A and CCK-B receptors: RNA sample was reverse transcribed into first strand complementary DNA (cDNA) using the Revert Aid H minus First Strand cDNA synthesis kit, Cat No. - K1632 (Fermentas Life Sciences, Canada). 20 µl of cDNA was prepared of each sample by using MJ Research, PTC 200 DNA Engine using 1 cycle of 60 min at 42°C and 1 cycle of 10 min at 70°C.

Real Time-PCR was performed on first strand cDNA (2.0 µl) using 1 cycle of 20 sec at 95°C, 35 cycles of 20 sec at 95°C, 20 sec at 58°C, 30 sec at 72°C and 1 cycle of 1 min at 95°C, 30 sec at 55°C, 30 sec at 95°C, using Stratagene Mx 3000P.

Ct value was obtained of each sample and ΔCt was calculated.

Ct = threshold Cycle, PCR cycle at which an increase in reporter fluorescence above a baseline signal is first detected (cycle when fluorescence crosses the threshold).

Calculation

ΔCt (sample) = Ct (Target) - Ct (Reference)

Where Target – CCK-A and CCK-B receptor, Reference-β-actin

The PCR products were electrophoresed on 2% agarose Tris-boric acid EDTA gel containing ethidium bromide (3 µl from 10 mg/ml stock in a volume of 35 ml).

The PCR amplification primers for the CCK-A and CCK-B genes were chosen (LifeTech AuPre P oligos, USA) and were based on the gene sequences published by de Weerth and colleagues [13] and Pisegna and associates [14]. The CCK-A fragment was of 320 base pair (bp) while CCK-B and β-actin fragment was of 430 bp and 410 bp respectively [13,14]. Following primers were used:

CCK-A primer pair, forward: 5´CCTACGACACCGCCTCCGC3´,

reverse: 5´TCCGTTCTTTCTTCTCTGCCTCCT3´.

CCK-B primer pair, forward: 5´ACCCCAACGACAGGAAAA-GGT3´,

reverse: 5´TTTGGGAAGGAAGGAGA-GGGC3´.

β-actin primer pair, forward: 5´CGACAGCAGTTGGTTGGAGC3´,

reverse: 5´GGTCTCAAGTCAGTGTACAG3´

STATISTICAL ANALYSIS

SPSS version 17 software was used for statistical analysis. Following tests were applied. Mean and Standard Deviation, Chi-square test for comparing two qualitative values, and ANOVA test for comparing multiple values were carried out {and if $p<0.05$ than Dunnett-C test, otherwise Tukey's test}.

RESULTS

Total 49 cases were recruited; 4 patients of cancer gall bladder were in advanced inoperable stage. In these cases only blood was taken for CCK receptors analysis. Thus, CCK receptors analysis was completed in 45 gall bladder tissue samples.

The mean age of the subjects was 45.12±13.19 years with a significant difference in the ages between cholelithiasis and CA gallbladder groups ($p<0.001$). The male (n=16) and female (n=33) ratio was 1:2. There was no significant difference in the haematological parameters and coagulation profile among patients and control subjects [Table/Fig-1]. However, significant difference was seen in the total ($p=0.025$) and conjugated serum bilirubin ($p=0.024$) levels between cholelithiasis and CA gallbladder groups [Table/Fig-1].

The preoperative ultrasonic findings of the subjects have been described in [Table/Fig-2]. There was significant ($p=0.001$) difference in the wall thickening between normal and patients of cholelithiasis and CA gallbladder. Intrahepatic biliary radical dilatation was present in one subject each of normal gall bladder and cholelithiasis as against 13 subjects having CA gallbladder ($p=0.000$) [Table/Fig-2].

The gallbladder tissue samples of all normal subjects and patients of gall bladder CA showed RNA expression whereas only 84.2% (n=16/19) patients in the cholelithiasis group found to have RNA expression. Both types of receptors, CCK-A and CCK-B, were expressed in each subject. It was further observed that there was increased expression of CCK-B receptors in about 2/3rd cases in all groups but the difference did not rise to significance ($p= 0.27$) [Table/Fig-3].

In blood samples of normal gall bladder subjects RNA expression was seen in all cases. But RNA expression was seen in 78.9% and 86.3% patients of cholelithiasis and CA gall bladder respectively. There was expression for both CCK-A and CCK-B receptors but unlike gall bladder tissue the expression of CCK-A was more (52.6% to 86.6%) in the blood compared to CCK-B receptors though statistically non-significant ($p= 0.17$) [Table/Fig-4]. Housekeeping gene (β-actin), which acted as control for the test procedure, was expressed in all patients where RNA and CCK receptors expressed. This confirmed the quality of the testing for expression of the CCK receptors.

DISCUSSION

Cholecystokinins are G protein-coupled receptors which are widely distributed in the body. Till now 2 types of CCK receptors, CCK-A and CCK-B, have been well studied. CCK-C, another Cholecystokinin receptor, has also been described recently [13]. Though these receptors are universally present in the body yet most of the studies have been done only on GIT.

In the stomach CCK-B receptors are predominantly expressed in fundic mucosa, corresponding to the location of parietal cells [14-16]. The CCK-A receptors which are present in the gastric muscle cause delay in gastric emptying [16,17]. In pancreas CCK-B receptors are predominantly expressed in exocrine pancreas and cause pancreatic enzyme secretion [18].

Parameters	Total (n=49)	Normal Gallbladder (n=8)	Cholelithiasis (n=19)	CA Gallbladder (n=22)	p value
Age (years)	45.12±13.1	43.25±15.3	37.42±14.5	52.43±5.1	0.001*
Haemoglobin (gm %)	12.25± 1.7	11.92± 1.5	12.99± 1.6	11.74±1.6	0.05
TLC (cell/mm^3)	8185.7± 2363.7	8862.5±4330.6	7863.1±1692.8	8218.1±1947.8	0.61
Platelet Count (lacs/mm^3)	2.64±0.50	2.76± 0.56	2.73±0.52	2.53±0.47	0.73
ESR (mm in 1st hr)	12.40± 5.1	15±8.2	11.49±5.1	12.23±3.4	0.27
Bleeding time (min.)	2.44±0.3	2.3±0.2	2.5±0.4	2.4±0.1	0.46
Clotting time(min.)	5±0.5	4.6±0.3	5.1±0.5	5.1±0.5	0.07
Prothrombin time (sec.)	13.4±0.8	13.2±0.7	13.2±0.6	13.7±0.9	0.09
S. Bilirubin (Total) (mg/dl)	1.33±1.5	0.96±0.9	0.76±1.2	1.96±1.6	0.025*
S. Bilirubin (Conj.) (mg/dl)	0.74±0.98	0.47±0.6	0.38±0.86	1.16±1.0	0.024*
S. SGOT (IU/L)	91.38±145.6	25.96±15.1	33.11±23.38	165.4±193.5	0.004*
S. SGPT (IU/L)	88±121.1	42.9±32.7	36.22±24.3	149.2±159.8	0.004*
S. Alkaline Phosphatase (IU/L)	355.4±540.3	140.6±71.6	240.2±555.3	532±582	0.1
S. Protein (g/dl)	6.84±0.5	6.99±0.6	7.05±0.4	6.59±0.5	0.017*
S. Albumin (g/dl)	4.05±0.6	4.2±0.3	4.26±0.6	4.26±0.6	0.07
S. Creatinine (mg/dl)	1.13±0.22	1.22±0.33	1.10±0.22	1.12±0.19	0.48
B. Urea (mg/dl)	27.06±8.26	33±10.98	26.06±8.72	25.77±5.86	0.08
S. Na+ (mmol/l)	138.9±2.95	139.38±2.13	138.95±2.64	138.68±3.51	0.85
S. K+ (mmol/l)	3.93±0.38	4.02±0.57	4.02±0.25	3.8±0.38	0.13

[Table/Fig-1]: Clinical profile of subjects
* indicates the values are statistically significant
TLC- Total Leucocyte count, Na+- Sodium, K+- Potassium

Parameters	Normal Gallbladder (n=8)	Cholelithiasis (n=19)	CA Gallbladder (n=22)	p value
Gallbladder wall thickening	0	13	16	0.001*
Gallbladder mass	0	0	22	Constant
Intrahepatic biliary radical dilatation	1	1	13	0.000*
Contiguous liver spread	0	0	4	0.06
Regional lymph node involvement	1	0	8	0.019*
Non-Contiguous liver spread	0	0	0	Constant
Ascites/ intraperitoneal collection	2	0	2	0.11
Single stone	0	3	5	0.000*
Multiple stones	0	16	14	
No stone	8	0	3	

[Table/Fig-2]: USG features
* indicates the values are statistically significant

Subjects	RNA	β-actin	Increased expression of CCK-A Receptors*	Increased expression of CCK-B Receptors*
Normal (n=8)	8	8	3 (37.5%)	5 (62.5%)
Gallstone (n=19)	16	16	7 (43.7%)	9 (56.3%)
CA gallbladder (n=18)^	18	18	5 (27.7%)	13 (72.3%)

[Table/Fig-3]: Cholecystokinin (CCK) receptors in gallbladder tissue samples
p = 0.27; * Both receptors expressed but one was more than other, ^ in four patients gall bladder tissue not obtained as they were inoperable

Subjects	RNA	β–actin	Increased expression of CCK A Receptors*	Increased expression of CCK B Receptors*
Normal (n=8)	8	8	5 (62.5%)	3 (37.5%)
Gallstone (n=19)	15	15	13 (86.6%)	2 (13.4%)
CA gallbladder (n=22)	19	19	10 (52.6%)	9 (47.4%)

[Table/Fig-4]: Cholecystokinin (CCK) receptors in blood sample
p= 0.17; * Both receptors expressed but one was more than other

CCK receptors in the human gallbladder have been extensively studied. CCK induces gallbladder contraction via activation of CCK-A receptor located on smooth muscle cells [13]. Both *in vivo* and *in vitro* binding assays have shown CCK receptors with high affinity in the human gallbladder [9,19]. Impaired gallbladder emptying has been reported in the patients with cholesterol gall stones, resulting from the defect in CCK-A receptor in the gallbladder [20].

Several studies on gallbladder contraction in patients with gallstones in response to direct or indirect stimulation of CCK release, have demonstrated increased or decreased sensitivity to this hormone. Two distinct groups of patients with gallstones have been identified; patients in whom gallbladder motility is depressed; and patients in whom gallbladder motility is unaltered inspite of diminished release of CCK. This suggests that gallbladder sensitivity to circulating CCK is increased in later group of patients suffering from cholelithiasis [21]. A mixture of both subsets of patients of gallstone in various studies may be the reason for differing results.

Von Schrenck et al., [22] reported that only CCK-A receptors were localized in the human gallbladder muscle. Another study showed that CCK-A receptor was more concentrated in human gallbladder though CCK-B receptors were also present [9]. However, we found that both CCK-A and CCK-B receptors were expressed, with overall predominance of CCK-B receptors in healthy as well diseased gallbladder tissue samples. Our observations may be explained by the fact that we studied whole gallbladder wall including mucosa, submucosa, muscle and serosa where spatial expression of CCK-A and CCK-B receptors may differ in different regions.

On comparing predominance of CCK-A receptor expression in the gallbladder tissue irrespective of disease state in our study it was found that, though not significant (p=0.27), there was higher expression of CCK-A receptor (44%) in Cholelithiasis group and decreased expression of CCK-A receptors in CA gallbladder group (27%) as compared to normal group (37%). Recent study showed that young patients suffering from gallbladder cancer has higher expression of CCK-A receptors but further studies on large sample required [23]. Studies show expression of CCK-B receptors in blood mononuclear [24] and polymorphonuclear cells [25]. Unlike gallbladder tissue the expression of CCK receptors in the blood was in favour of CCK-A receptors but pattern was similar; it was

more in the patients of cholelithiasis and less in cancer gallbladder in comparison to normal gallbladder tissue. So, no definite conclusion can be made about the expression of CCK-A and CCK-B receptors in different gall bladder diseases. To the best of our understanding not enough studies are available for comparison of results of our study. In fact much is left to be learnt about physiological functions and control and pathological alterations of CCK receptors in normal and diseased gallbladder before any conclusion can be drawn about relative importance of CCK-A and CCK-B receptors in the causation or effect of the disease.

LIMITATION

The limitation of our study was that besides being a preliminary study on examining the expression of cholecystokinin receptors in the gallbladder tissue and blood in diseased gallbladder, the small sample size and inability to study other parameters like genetic makeup, different food habits, and racial differences in these subjects further compromised scope of the work.

CONCLUSION

Our study showed that both CCK-A and CCK-B receptors were expressed in the gallbladder tissue and blood of patients with normal gallbladder, and gallbladder with cholelithiasis and CA. There was higher expression of CCK-A receptors in patients of cholelithiasis and decreased expression of CCK-A receptors in patients of CA gallbladder as compared to normal gallbladder although it did not rise to statistical significance.

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