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Original Research Article

Cholecystokinin Type-A Receptor in Gall Bladder Lesions: A Correlative Study

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Abstract: Carcinoma gallbladder is one of the most common malignancies of gastrointestinal tract. It is highly aggressive and incurable disease representing most common malignancy of biliary tract with a three folds higher incidence in females. Gall bladder cancer shows striking geographical predilections in its incidence, with highest figures found in India and Chile and relatively low level in many western countries. Regulatory peptide receptors have attracted interest of oncologists as a new promising approach for cancer pathology, imaging and therapy. Although cholecystokinin (CCK) is a potent modulator of gallbladder contractility and plays a potential role in pancreatic carcinogenesis through CCK type-A receptor (CCKAR), its role in gallbladder cancer (GBC) is still unknown and immunohistochemical detection of CCKAR in the gallbladder has not yet been reported. The aim is to investigate the expression profile of CCKAR in GBC and Chronic cholecystitis. This case-control study included 100 samples: 50 from GBC and 50 from Chronic cholecystitis. Expression of CCKAR was analyzed by immunohistochemistry. The results were statistically correlated with disease history including age, sex and differentiation. CCKAR was positive in 21/50 (42.0%) of chronic cholecystitis and 38/50 (76.0%) of GBC samples. 21 of the 38 (55.3%) CCKAR-positive GBC samples showed strong expression. There was a significant difference in CCKAR expression between chronic cholecystitis and GBC. CCKAR expression was significantly increased in GBC compared to chronic cholecystitis. Moreover, CCKAR expression was associated with the degree of tumor differentiation, i.e., less expression in poorly-differentiated tumors. So, it has future prognostic and therapeutic implications in the management of GBC.
Keywords: cholecystokinin, cholecystokinin type-A receptor, gallbladder cancer, chronic cholecystitis

INTRODUCTION

Gall bladder cancer is most common malignancy of biliary tract with a three folds higher incidence in female [1]. Gall bladder cancer shows striking geographical predilections in its incidence, with highest figures found in India and Chile but a relatively low level is seen in many western countries [2]. In northern India, especially along the gangetic belt [3] there is a high incidence. The incidence of carcinoma gallbladder varies widely in different geographic regions, and racial and ethnic groups. In India, it is the most common form of biliary malignancy and third most common carcinoma of the digestive tract in Eastern UP and Western Bihar [4] and fifth most common gastrointestinal carcinoma in women [5-7]. Cholelithiasis, especially untreated chronic symptomatic gallstones, with inflammation is one of the main risk factors of gallbladder cancer. Most of the gall bladder carcinomas have regional disease or distant metastases at presentation with poor prognosis

The cholecystokinin (CCK) and gastrin families of peptides act as hormones and neuropeptides on central and peripheral CCK receptors to mediate secretion and motility in GIT in physiological response to a normal meal. CCK-A receptor, found predominantly in the GI system and selective areas of the CNS, have high affinity for CCK and the

nonpeptide antagonist L-364,718. The physiological functions of gall bladder are done through different receptors. The main neurohormonal mechanisms regulating the motility of the gallbladder are the vagus and splanchnic nerves and the hormone CCK. The sub-types of receptors for CCK in the human pancreas and gallbladder are different. The human pancreas predominantly expresses CCK-B receptor, whereas CCK-A receptor are localized in the human gallbladder muscle [8].

It is known that gallbladder has high concentration of CCK-A receptor [9]. CCK-A receptor does not modulate the susceptibility of cancer gallbladder [10]. But their role in gallbladder malignancy and other gall bladder lesions 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 severity and thereby increasing ailments of patients through social, economical, financial and emotional stigma. This may dictate for measures to be taken in developing new screening or therapeutic strategies for developing gall bladder pathologies at the earliest.

The aim of study was to evaluate and compare the expression of CCK-A receptor in non-neoplastic and neoplastic (different histopathological grades) lesions of gall bladder that may provide an ultimate hope in its prognostic evaluation.

MATERIALS AND METHODOLOGY

Patients

This case-control study included patients undergoing surgery for gall bladder carcinoma and gall stone disease from November 2014 to May 2016 in the Department of Pathology in collaboration with Department of Surgery, Era's Lucknow Medical College and Hospital, Lucknow. The sample sizes were calculated as 50 for each group.The study population comprised of patients undergoing surgical procedure for gall bladder diseases either through open or laparoscopic procedures. Specimen were subjected to routine histopathological processing, diagnosed cases of adenocarcinoma of gall bladder were taken and grading was done as well, moderately and poorly differentiated carcinoma. Non-neoplastic cases which includes chronic cholecystitis with or without cholelithiasis were taken as control. Patients with double malignancy, immunodeficiency diseases or any other associated chronic debilitating disorder which is likely to interfere with detection of marker were excluded.

Immunohistochemistry

3-4 μm sections from paraffin embedded blocks were cut and placed on polylysine-coated slides and used for immunohistochemical staining. Primary antibody and a secondary kit used for detection of CCK-A receptor (CCK-A receptor (H-60) antibody; sc-33220) were from Santa Cruz, and the Super Sensitive Link-Lable IHC Detection System (QD000-5L) was from BiogGenex, San Ramon, CA, USA. Briefly, all sections were dewaxed and rehydrated in xylene and graded alcohol, and placed under slow running tap water for 15 minutes followed by citrate buffer (pH 6.0) retrieval by the microwave method.The sections were allowed to cool at room temperature and washed 3 times with Tris buffer (TBS, pH 7.6).Then they were incubated with peroxidase block for 20 minutes to check internal peroxidase activity. After washing with TBS, these sections were incubated with power block for 15 minutes to block nonspecific staining. Excess power block was removed and then the sections were immediately incubated with primary antibody at1:200 dilution in TBS overnight at 4 °C. After washing with TBS, the sections were incubated with multilink for 30 minutes and washed with TBS followed by secondary antibody incubation again for 30 minutes. After washing, color was developed using diaminobenzidine (DAB) as the chromogen. Finally, slides were washed and counterstained with Harris hematoxylin. For positive control, a section known to stain positively on the gallbladder muscle layer component was included in each batch of staining, and for negative control primary antibody was replaced with TBS.

Evaluation of IHC staining pattern

The results were evaluated quantitatively as well as qualitatively according to the intensity of staining pattern by the scoring system used for breast cancer, as there is no standard scoring system for gallbladder cancer [11]. Intensity of staining was graded as negative (0), weak (1), moderate (2) or strong (3). The percentage of cells showing staining was graded as: none (0), 1 (<1%), 2 (1%-10%), 3 (11%-33%), 4 (34%-66%) or 5 (>66%).Total staining score was calculated by adding the intensity score and the percentage score as negative (0), weak/+ (2), moderate/2+ (3-5), or strong/3+ (6-8).

RESULTS

Out of 100 subjects enrolled in the study, a total of 50 neoplastic were taken as cases and whereas remaining 50 non-neoplastic controls which comprised of chronic cholecystitis were taken.(Figure 1:Gross of Chronic cholecystitis with cholelithiasis). Among

neoplastic cases, all adenocarcinomas were taken. Age of patients ranged from 30 to 70 years. Overall mean age of patients was 48.42±12.26 years and majority of patients were below 40 years of age (n=33; 33%). Age of patients in neoplastic group ranged from 30 to 70 years with a mean age of 55.88±8.93 years. In Non-neoplastic group, age of patients ranged from 30 to 70 years with a mean age of 40.96±10.55 years. On evaluating the data statistically, the difference between two groups was found to be significant (p<0.001). All cases except only 18 (36%) in Neoplastic group and 22 (44%) in Non-neoplastic group were females. Statistically, no significant difference between two groups was observed with respect to gender (p=0.414).

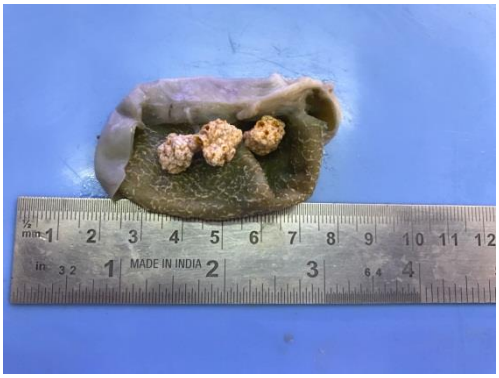

Fig-1: Chronic cholecystitis with cholelithiasis

CCKAR expression, by immunostaining showed 76% positive cases while in non-neoplastic only 42% cases showed positivity. On statistical analysis, there was a significant difference in CCKAR expression between both the groups. Out of 50 neoplastic (adenocarcinoma) subjects enrolled in the study, 66% were well-differentiated, [Figure 2:Gross of Well differentiated adenocarcinoma (Papillary) Gall Bladder], 14% cases were diagnosed as moderately differentiated and 20% as poorly differentiated adenocarcinomas respectively. The CCKAR positivity was maximum in Well Differentiated and was minimum in Poorly Differentiated adenocarcinomas, while a statistically significant difference occurred between Poorly Differentiated vs Moderately Differentiated, and Poorly Differentiated vs Well Differentiated samples. Interestingly, the overexpression of CCKAR was significantly associated mainly with Well Differentiated and Moderately Differentiated samples in comparison to Poorly Differentiated samples.

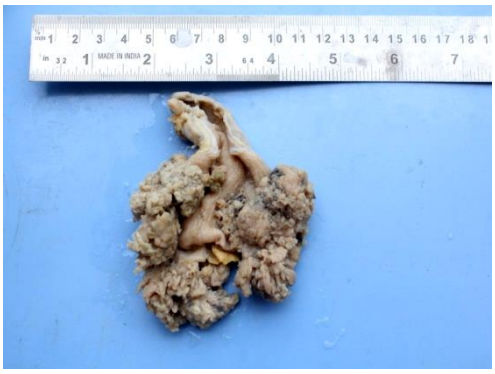

Fig-2: Well differentiated adenocarcinoma (Papillary) Gall Bladder

Out of 50 non neoplastic lesions 29 controls were scored 0 taken as negative,(Figure 3:Photomicrograph of Chronic Cholecystitis, H&E, 10X) & (Figure 4: Photomicrograph of Chronic cholecystitis , IHC for CCKAR showing negative staining 40X), 17 controls were scored 1+ taken as weak positive while only 4 controls were scored 2+ taken as moderately positive and none scored 3+ taken as strongly positive. However, in the 50 neoplastic lesions, 33 were well differentiated adenocarcinomas. (Figure 5: Photomicrograph of Well differentiated adenocarcinoma Gall Bladder, H&E, 10X) 18 out of 33 well differentiated adenocarcinomas were scored 3+ taken as strongly positive for CCKAR.10 out of 33 were scored as 2+ taken as moderately positive (Figure 6: Photomicrograph of Well differentiated adenocarcinoma Gall Bladder, IHC for CCKAR showing cytoplasmic positivity) &(Figure7: Photomicrograph of Well differentiated adenocarcinoma Gall Bladder,IHC for CCKAR showing cytoplasmic positivity, 40X), only 1 was scored as 1+ taken as weakly positive and remaining 4 were scored 0 taken as negative.

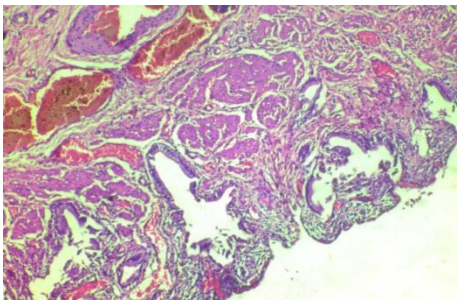

Fig-3: Photomicrograph of Chronic cholecystitis (H&E, 10X)

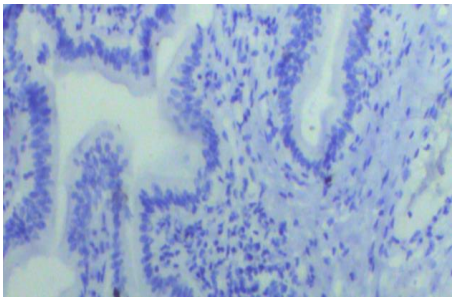

Fig-4: Photomicrograph of Chronic cholecystitis (IHC for CCKAR showing negative staining, 40X)

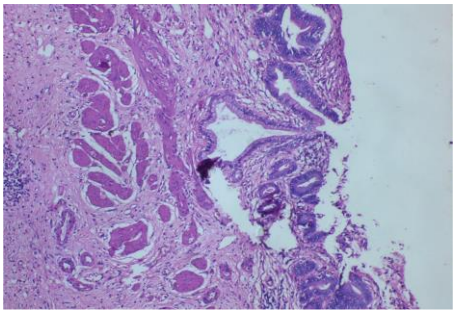

Fig-5: Photomicrograph of Well differentiated adenocarcinoma Gall Bladder (IHC for CCKAR showing cytoplasmic positivity, 10X)

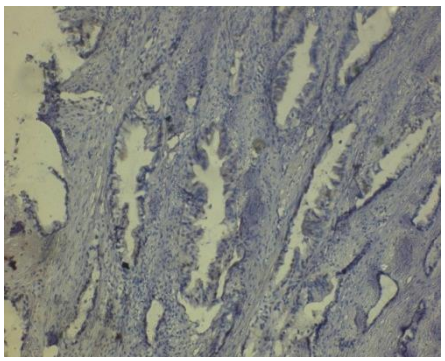

Fig-6: Photomicrograph of Well differentiated adenocarcinoma Gall Bladder (H&E, 10X)

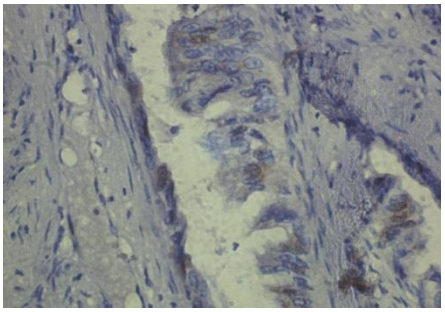

Fig-7: Photomicrograph of Well differentiated adenocarcinoma Gall Bladder (IHC for CCKAR showing cytoplasmic positivity, 40X)

Out of 7 moderately differentiated adenocarcinomas 5 were scored 2+ taken as moderately positive, 1 was scored 1+ taken as weakly positive and only 1 was scored 0 taken as negative for CCKAR.

In poorly differentiated adenocarcinomas out of total 10 cases, 3 were scored as 3+ taken as strongly positive), and 7 were scored 0 taken as negative.

However, none out of total cases categorized as moderately and poorly differentiated adenocarcinomas showed strong immunostaining for marker.

Expression of CCKAR was hence statistically significant between well differentiated and poorly differentiated adenocarcinoma (p<0.001)and also between moderately and poorly differentiated adenocarcinomas (p<0.048) (Table 1, Figure 8).

Table 1: CCKAR expression in different grades of Gall bladder lesion

S.no.	Total cases N=100	CCKAR Score Negative (Score 0)	CCKAR Score Weak(Score1+)	CCKAR Score Moderate(Score2+)	CCKAR Score Strong(Score3+)
1.	Non-neoplastic (N=50)	29 (58%)	17(34%)	4(8%)	0
2.	Neoplastic (Adenocarcinoma) (N=50)				
•	Well Diff. (N=33)	4 (12.1%)	1 (3%)	10 (30.3%)	18 (54.5%)
•	Moderately Diff. (N=7)	1 (14.3%)	1 (14.3%)	5 (71.4%)	0
•	Poorly Diff. (N=10)	7 (70%)	0	3 (30%)	0

Applied $\chi 2$ test for significance.
*Well Diff. vs Moderately Diff.- p value: 0.048(S), **Well Diff. vs Poorly Diff.- p value: <0.001(S), ***Moderately Diff. vs Poorly Diff.- p value: 0.059

Total staining score was calculated by adding the intensity score and the percentage score as negative (0), weak/+ (2), moderate/2+ (3-5), or strong/3+ (6-8).

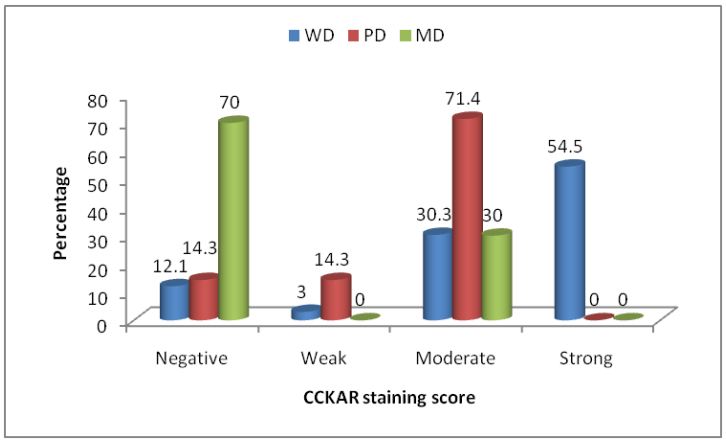

Fig-8: CCKAR expression in different grades of Gall bladder lesions

CCKAR expression showed a mean of 4.42 in neoplastic and a mean of 1.78 in non-neoplastic groups. Standard deviation was 2.408 for neoplastic and 1.250 for non-neoplastic groups. On comparing the data statistically, CCKAR expression was significantly higher in neoplastic group as compared to non neoplastic group (p<0.001) (Table 2).

Table 2: CCKAR expression in neoplastic and non neoplastic group

Group	N	Mean	Std. Deviation
Neoplastic	50	4.42	2.408
Non neoplastic	50	1.78	1.250

p value= <0.001(S)

DISCUSSION

Gallbladder is the common site for malignancy and is the fifth most common site among gastrointestinal tract-related organs [12-15]. Carcinoma of the gallbladder is more frequent in females than males (3 to 4:1 ratio); over 90% of the patients are 50 years of age or older at the time of diagnosis. Diseases of the gallbladder commonly manifest as gallstones and gallbladder cancer [16]. Gallstones are a common occurrence and there are a number of surgical modalities available for treatment of gallstone. A definite epidemiologic parallel exists between gallbladder carcinoma and cholelithiasis, but the pathogenetic relationship between them remains controversial [17-20]. Although, gallbladder cancer is a rare yet it is associated with lethal malignancy with marked ethnic and geographical variations. In general, GBC is the most aggressive of the biliary cancers with the shortest median survival duration [21]. Owing to vague presentation their diagnosis commonly occurs at an advanced stage.

With this background the present study was carried out to correlate and evaluate the prognostic significance of the expression of CCKAR in neoplastic and non-neoplastic gall bladder lesions.

CCK is one of the longest known hormones released from endocrine cells of the small intestine. Initially it was identified as an important factor for controlling gallbladder motility and pancreatic enzyme secretion [22]. Increasing evidence has demonstrated a trophic effect of CCK on the pancreas, gastrointestinal mucosa and epithelial cells of the gallbladder [23, 24]. All these physiological actions of CCK are mediated by G-protein coupled receptors, either the CCKAR or the CCCKBR, which have 48% structural homology, though CCKAR has higher affinity for sulphated CCK than gastrin/nonsulphated CCK. CCKARs are predominantly distributed in the gallbladder, pancreas and brain while CCK-B/ gastrin receptors are present in the gut mucosa and brain.

Series of studies by Kano M *et al.,* [25] in 2002 had shown that formation of cholesterol-supersaturated bile in subjects with cholesterol gallstone disease is causatively related to decreased gallbladder contractility and mucin hypersecretion by the gallbladder. Supersaturated bile may modify the composition of gallbladder membranes so that the transduction of smooth muscle regulatory signals is impaired, and it may enhance the inflammation-induced mucin secretion by the gallbladder. It showed that CCKAR has strong association with gall stone formation which may indirectly lead to carcinogenesis.

A study conducted by Norikazu *et al.*, [26] in 2003 was intended to identify the role of CCK-AR on gallstone formation and deteriorated gallbladder contraction due to a lack of CCK-AR favored gallstone formation after the middle age of life.

Srivastava A *et al.*, [27] in their study showed similar findings that frequency of the A1A1 genotype of CCK-AR was significantly higher in gallstone patients than healthy individuals and the results suggest that the A1A1 genotype of CCK-AR is an independent genetic risk factor for gallstone disease and does not modulate the susceptibility of gallbladder cancer.

CCKARs are expressed in a variety of human tumors, primarily in significant numbers of gastroenteropancreatic tumors, meningiomas, and neuroblastomas [28].

A Study done by Rai *et al.,* in 2011 demonstrated gradual increase in CCKAR expression from GSD to GBC. Since long-standing gallstones have been attributed to the pathogenesis of GBC [29, 30] it seems that aberrant CCKAR expression is associated with disease progression from cholelithiasis to early carcinoma. They demonstrated CCKAR positivity in 44.1% of GSD and 76.6% of GBC samples with a significant difference. The GSD showed mainly weak and moderate expression (43.3%). On the contrary, 70.8% of CCKAR-positive GBC cases revealed over-expression which is similar to our results. This over-expression may be due to up-regulation of CCKAR mRNA. They also performed immunoblotting in which they found that the level of CCKAR expression was significantly higher in GBC than in GSD. In present study, IHC of gall bladder specimens of both neoplastic and non-neoplastic lesions showed that 76% of neoplastic samples had over expression of CCKAR as compared to 42% of non-neoplastic samples. The findings of the study explained above corroborate with the findings of the present study.

Rai R *et al*., [31] in their study also showed similar finding as that of present study, in association of degree of tumor differentiation with CCKAR expression, i.e., high expression in well differentiated adenocarcinomas. Thus, it has future prognostic and therapeutic implications in the management of GBC. Present study showed higher expression of CCKAR of about 85% in well differentiated carcinomas but only 5% in poorly differentiated adenocarcinomas.

Study done by Hong Li-Xu *et al.,* in 2013 [32] in china investigated the associations between nine single nucleotide polymorphisms in *CCK* and *CCKAR* in a population-based case–control study, including 439 biliary tract cancer cases (253 gallbladder, 133 extrahepatic bile duct, and 53 ampulla of Vater cancer cases), 429 biliary stone cases, and 447 population controls in Shanghai, China. They found that women with the *CCKAR* rs1800855 AA genotype had an increased risk of gallbladder cancer compared with subjects with the TT genotype, and remained significant after Bonferroni correction. Their findings suggested that variants in the *CCKAR* gene may influence the risk of gallbladder cancer in women. In present study role of CCKAR was studied on tissue sections of neoplastic and non neoplastic lesions and it noted the over expression of CCKAR in neoplastic lesions of gall bladder.

However, **i**n the study conducted by Okada N *et al*., [33] in 1996 showed that reverse transcription-polymerase chain reaction (RT-PCR) was used to evaluate messenger RNA expression for CCK, gastrin, CCK-A receptor, and CCK-B/gastrin receptor in surgical specimens of gastric cancers and in normal antrum and body mucosa of the stomach. Their findings suggested a greater role for CCK and CCKAR in gastric cancers.

Finno K *et al.*, [34] in their study done in 2012 showed that the CCK-BR drives growth of pancreatic cancer; hence, interruption of CCK-BR activity could potentially be an ideal target for cancer therapeutics.

A similar study on " Functional significance of the cholecystokinin-C (CCK-C) receptor in human pancreatic cancer" by Smith J P *et al.,* [35] in 2004 showed that CCK-C receptor is functional and plays a crucial role in growth of human pancreatic cancer.

Their findings suggested a greater role for CCK-C receptor in pancreatic cancers.

Also a study conducted by Schulz S *et al*., [36] in 2005 showed that the presence of CCK1 receptors was rarely detected in human tumors except for carcinoids, insulinomas, pituitary adenomas, and meningiomas.

So, Overexpression of CCK receptor is definitely having association with some form of carcinogenesis. The findings of present study thus showed that positivity of CCKAR expression have a significant role in differentiating non neoplastic from neoplastic lesions of gall bladder. Moreover, these features also helped to differentiate amongst different histopathological grades of gallbladder cancer. CCKAR expression is also found in a myriad of gastrointestinal disorders. Detection of CCKAR in normal gall bladder and in epithelial dysplasia should be done to determine whether CCKAR expression progressively increases with the onset of changes in the gallbladder epithelium. So, coexpression of CCK, *in situ* detection of CCKAR, secondary signalling pathways linked to it and the mechanism of its up-regulation, should also be examined to explore the involvement of this receptor in the development and progression of GBC. There are limited studies evaluating the role of CCKAR showing a promising role in differentiating neoplastic from non-neoplastic lesions and differentiation of different histopathological grades of gall bladder carcinoma so, the evidence related with these associations needs an empirical validation for which further studies are recommended.

CONCLUSION

CCK is admittedly one of many regulatory peptides or hormones involved in GBC. Present study thus showed that positivity of CCKAR expression have a significant role in differentiating non-neoplastic from neoplastic lesions of gallbladder. Moreover, these features also helped to differentiate amongst different histopathological grades of gallbladder cancer. CCKAR is significantly overexpressed in GBC and encourages the inclusion of more peptides and hormones in future studies. In addition, the over-expression of CCKARs in most cases of GBC may suggest the use of receptor antagonists for tumor localization, clinical assessment, and/or receptor-based delivery of therapeutic agents (cytotoxic toxin linked to a specific ligand of these receptors) to treat GBC, thus providing a new avenue of research.

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