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A Study of Cyclin D1, E-cadherin, EGFR, HER- 2, Ki67, and p53 Tumor Marker Expressions in Neoplastic and Non -neoplastic Gall Bladder Lesions and their Clinico-pathological Correlation

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Abstract

Background: Worldwide gall bladder cancer (GBC) is known to be the commonest malignant tumour of the biliary tract. It is the most aggressive carcinoma of the biliary tract with short median survival from the time of diagnosis. The aggressive biologic behaviour of the carcinoma and non-availability of sensitive screening tests for early detection may be responsible for the poor prognosis associated with GBC.

Material and Methods: Patients diagnosed with neoplastic and non-neoplastic gallbladder lesions in the Department of Pathology, Subharti Medical College in India were included in the study. Gall bladder biopsies findings and the clinic-pathological data were compiled and different immune-markers diagnostic and therapeutic usefulness were studied .

Results: 24 (24.0%) of the cases were in the age group of 51-60 Years. In 38 (38.0%) of the cases the site of lesion was Fundus. 55 (55.0%) of cases had associated stones. 36 (36.0%) of them had P53: strong over expression, 40 (40.0%) of the them had ki67: strong over expression, 25 (25.0%) had EGFR: strong over expression whereas negative expression for Her2/Neu was found in 61 (61.0%) of the cases. Cyclin D1: moderate over expression was found in 27 (27.0%) of them and moderate over expression of E-cadherin were found in 22 (23.2%) cases.

Conclusions: Novel prognostic biomarkers could bring about the needed breakthrough in gall bladder cancer diagnosis and treatment. We conclude that the biomarkers studied by us may help in the identification of cases who may benefit tremendously from adjuvant and targeted therapies.

Key words: Cyclin D1, E-cadherin, EGFR, HER- 2, Ki67, p53, tumour marker, neoplastic , non –neoplastic, Gall bladder lesions.

Introduction

Gallbladder carcinoma (GBC) is an aggressive cancer of the biliary tract. It arises from the epithelia of the gallbladder and is has the ability to rapidly metastasize to nearby organs and distant lymph nodes. It also has the shortest median survival time when compared to other biliary tract cancers [1]. As the disease hardly presents a discrete set of clinical symptoms,

delayed diagnosis contributes strongly towards the bad prognosis usually associated with the disease [2]. The molecular mechanisms and underlying changes associated with the carcinogenesis of the gallbladder have not been fully understood. Chronic inflammation, dysplasia and adenoma among other factors have been noted to increase the risk of developing GBC [3-4]. With current treatment options offering only limited curative capacities, identifying and studying biomarkers with potential to speed up prognostics and having clinical applicability has become a priority. These biomarkers, apart from playing an important role in carcinogenesis, need to be specific for GBC and their levels should vary significantly enough to differentiate between malignant and benign conditions. Though several such biomarkers have been identified and used in diagnostic trials, the availability of disease-specific and highly sensitive markers for GBC is yet a task to be achieved. Isolation of such prognostic markers will help in understanding disease progression [5 ~~6~~]. Further, expression levels of these prognostic markers can be used to aid in the determination of the clinical course of action, patient response to therapy and the need for adjuvant therapy [~~7~~]. An ideal prognostic marker should be easily quantifiable with high sensitivity, specificity and its levels should significantly vary to be able to differentiate benign conditions from malignancy. A clinically applicable prognostic marker should be able to predict recurrence, survivability and need for adjuvant therapies.

Material and Methods

All consecutive patients diagnosed with neoplastic and non-neoplastic gallbladder lesions in the Department of Pathology, Subharti Medical College were included in the study between the year 2017 -2019. The hematoxylin and Eosin stained biopsies of gall bladder lesions were assessed and 100 cases were chosen as the sample for the study. And Immuno-markers Cyclin D1, E-cadherin, EGFR, HER- 2, Ki67, and p53 tumour were used to see their correlation between clinic-pthological features in the gall bladder lesion and study its therapeutic and diagnostic significance.

Results:

Table 1: Age/Gender of cases with Gall Bladder lesions

Age/Gender	Mean ± SD || Median (IQR) || Min-Max || Frequency (%)
Age	
<20 Years	3 (3.0%)
20-30 Years	8 (8.0%)
31-40 Years	10 (10.0%)
41- 50 Years	23 (23.0%)
51-60 Years	24 (24.0%)
61-70 Years	23 (23.0%)
>70 Years	9 (9.0%)
Gender	
Male	51 (51.0%)
Female	49 (49.0%)

3 (3.0%) of the cases had age <20 years. 8 (8.0%) of the cases had age between 20-30 years. 10 (10.0%) of the cases were in the age group of 31-40 years. 23 (23.0%) of the cases had age between

41- 50 years. 24 (24.0%) of the cases were in the age group of 51-60 years. 23 (23.0%) of the cases had age between 61-70 years. 9 (9.0%) of the cases had age more than >70 years.
51 (51.0%) of the cases in our study were males. 49 (49.0%) of the cases were female.

Table 2: Summary of findings on Clinico-Pathological Examination (CPE) of Gall Bladder Cases

CPE	Mean ± SD || Median (IQR) || Min-Max || Frequency (%)
Type of Gall Bladder Lesions	
Non-Neoplastic	50 (50.0%)
Neoplastic	50 (50.0%)
Site	
Fundus	38 (38.0%)
Body	28 (28.0%)
Neck	3 (3.0%)
Fundus and Body	13 (13.0%)
Fundus, Body and Neck	18 (18.0%)
Macroscopic Type (Gross)	
Grey White Irregular Mass	28 (28.0%)
Polyp	21 (21.0%)
Wall Induration	27 (27.0%)
Ulcers	5 (5.0%)
Calcification	10 (10.0%)
Perforation	9 (9.0%)
Gall Bladder Wall Thickness	
>3 mm	67 (67.0%)
<3 mm	33 (33.0%)
Associated Stones	
Stones Present	55 (55.0%)
Stones Absent	45 (45.0%)
Type of Gall Bladder Lesions	
Neoplastic Lesion	
Adenocarcinoma	9 (17.6%)
Papillary Adenocarcinoma	7 (13.7%)
Adenocarcinoma, Intestinal Type	11 (21.6%)
Mucinous Adenocarcinoma	8 (15.7%)
Adenosquamous Carcinoma	4 (7.8%)
Undifferentiated Carcinoma	5 (9.8%)
Carcinoma, Not Otherwise Specified	7 (13.7%)
Non-Neoplastic Lesion	
Acute Cholecystitis	3 (6.1%)
Chronic Cholecystitis	8 (16.3%)
Acute On Chronic Cholecystitis	3 (6.1%)
Chronic Cholecystitis with Cholesterolosis	7 (14.3%)
Eosinophilic Cholecystitis	2 (4.1%)
Xanthogranulomatous Cholecystitis	3 (6.1%)
Porcelain Gall Bladder	3 (6.1%)
Empyema Of Gall Bladder	4 (8.2%)
Adenomatous Hyperplasia	4 (8.2%)

CPE	Mean ± SD || Median (IQR) || Min-Max || Frequency (%)
Papillary Hyperplasia	3 (6.1%)
Pyloric Gland Metaplasia	3 (6.1%)
Intestinal Metaplasia	3 (6.1%)
Dysplasia	3 (6.1%)
Histological Grade	
Benign Lesions	49 (49.0%)
GX: Cannot Be Assessed	0 (0.0%)
G1: Well Differentiated	15 (15.0%)
G2: Moderately Differentiated	23 (23.0%)
G3: Poorly Differentiated	1 (1.0%)
G4: Undifferentiated	12 (12.0%)
Pathologic Staging (pTNM)	
pTx: Cannot Be Assessed	49 (55.7%)
pT0: No Evidence Of Primary Tumor	0 (0.0%)
pTis: Carcinoma In Situ	0 (0.0%)
pT1: Tumor Invades Lamina Propria Or Muscular Layer	9 (10.2%)
pT1A: Tumor Invades Lamina Propria	7 (8.0%)
pT1B: Tumor Invades Muscle Layer	0 (0.0%)
pT2: Tumor Invades Perimuscular Connective Tissue; No Extension Beyond Serosa Or Into Liver	10 (11.4%)
pT3: Tumor Perforates Serosa ,Liver ,Adjacent Organ Or Structures	’13 (14.8%)
pT4: Tumor Invades Main Portal Vein Or Hepatic Artery Or Invades 2 Or More Extrahepatic Organs Or Structures	0 (0.0%)
Regional Lymph Nodes (pN)	
Benign lesions	49 (49.0%)
pNX: Cannot Be Assessed	0 (0.0%)
pN0: No Regional lymph Node Metastasis	1 (1.0%)
pN1: Metastases To Nodes Along The Cystic Duct, Common Bile Duct, Hepatic Artery, And/Or Portal Vein	1 (1.0%)
pN2: Metastases To Periaortic, Pericaval, Superior Mesentery Artery, And/Or Celiac Artery lymph Nodes	21 (21.0%)
No Nodes Submitted Or Found	28 (28.0%)
Distant Metastasis (pM)	
Benign lesions	49 (49.0%)
Not Present	10 (10.0%)
Distant Metastasis	41 (41.0%)

In 38 (38.0%) of the cases site for gall bladder lesion was fundus. In 28 (28.0%) of the cases the site of gall bladder lesion was body and 18 (18.0%) of the cases had site: fundus, body and neck respectively.

In 28 (28.0%) of the cases the macroscopic type (gross) finding of gall bladder lesion was grey white irregular mass, 27 (27.0%) of the cases had macroscopic type (gross) presentation as wall induration, in 5 (5.0%) of the cases macroscopic type (gross) were ulcers. 10 (10.0%) of the cases had macroscopic type (gross): calcification.

In 67 (67.0%) of the cases gall bladder wall thickness was more than >3 mm. 33 (33.0%) of the cases had gall bladder wall thickness less than <3 mm.

55 (55.0%) of the cases had associated stones. The diagnosis of gall bladder lesion was as follows: adenocarcinoma in 9 (9.0%), papillary adenocarcinoma type in 7 (7.0%) , adenocarcinoma, intestinal type in 11 (11.0%) , mucinous adenocarcinoma type in 8 (8.0%) , adenosquamous carcinoma in 4 (4.0%) of the cases , undifferentiated carcinoma in 5 (5.0%) of the cases , carcinoma, not otherwise specified in 7 (7.0%) , acute cholecystitis in 3 (3.0%) of the cases , chronic cholecystitis in 8 (8.0%) , acute on chronic cholecystitis in 3 (3.0%) of the cases , chronic cholecystitis with cholesterolosis in 7 (7.0%) of the cases , eosinophilic cholecystitis in 2 (2.0%) of the cases , xanthogranulomatous cholecystitis in 3 (3.0%) of the cases , porcelain gall bladder in 3 (3.0%) of the cases , empyema of gall bladder in 4 (4.0%) of the cases , adenomatous hyperplasia in 4 (4.0%) , papillary hyperplasia in 3 (3.0%) of the cases , pyloric gland metaplasia in 3 (3.0%) , intestinal metaplasia in 3 (3.0%) and dysplasia in 3 (3.0%) of the cases respectively.

15 (15.0%) of the cases had histological grade: g1: well differentiated. 23 (23.0%) of the cases had histological grade: g2: moderately differentiated. 1 (1.0%) of the cases had histological grade: g3: poorly differentiated. 12 (12.0%) of the cases had histological grade: g4: undifferentiated.

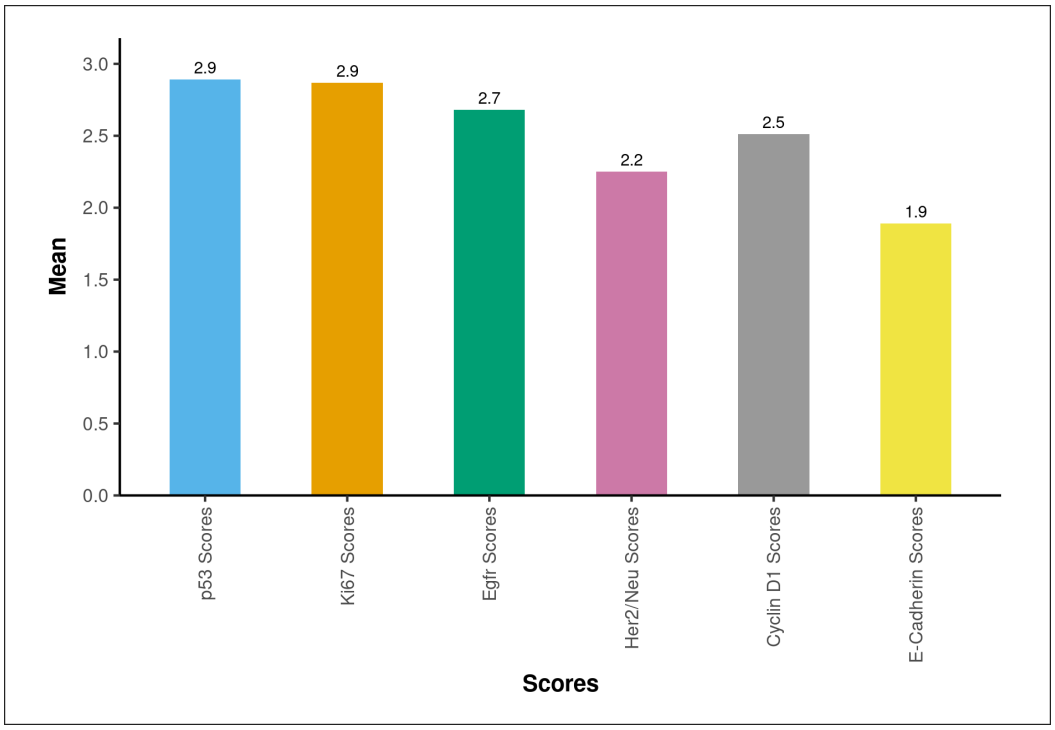
9 (10.2%) of the cases had pathologic staging (ptnm): pt1: tumor invades lamina propria or muscular layer. 7 (8.0%) of the cases had pathologic staging (ptnm): pt1a: tumor invades lamina propria. 0 (0.0%) of the cases had pathologic staging (ptnm): pt1b: tumor invades muscle layer. 10 (11.4%) of the cases had pathologic staging (ptnm): pt2: tumor invades perimuscular connective tissue; no extension beyond serosa or into liver. 13 (14.8%) of the cases had pathologic staging (ptnm): pt3: tumor perforates serosa , liver ,adjacent organ or structures. 0 (0.0%) of the cases had pathologic staging (ptnm): pt4: tumor invades main portal vein or hepatic artery or invades 2 or more extrahepatic organs or structures.

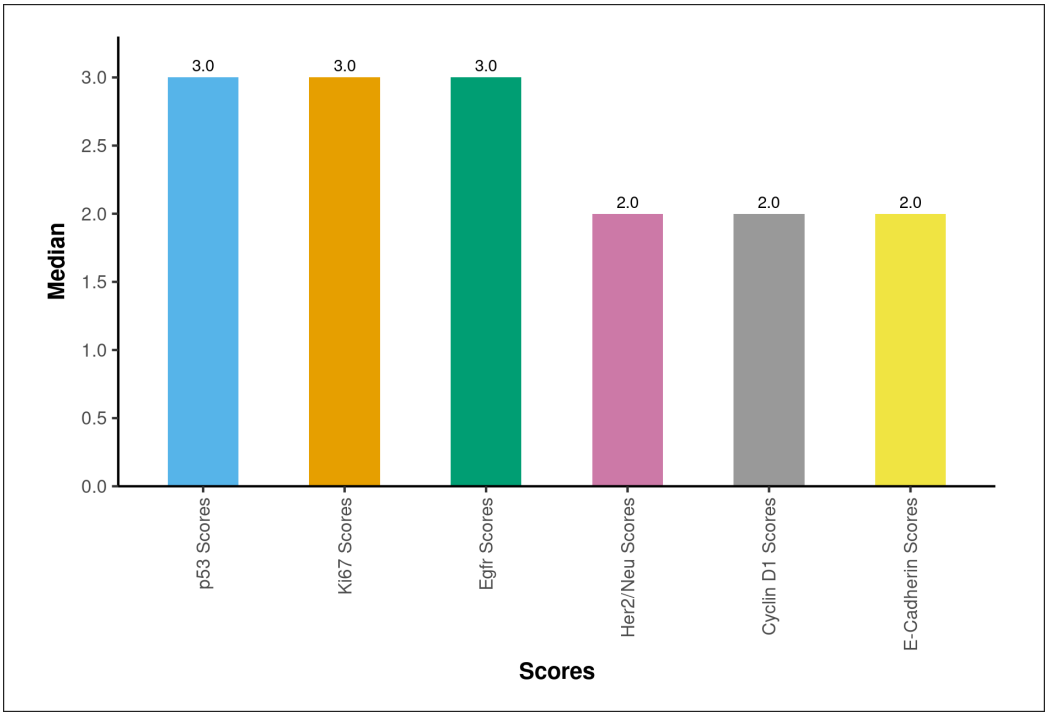
21 (21.0%) of the cases had regional lymph nodes (pn): pn2: metastases to periaortic, pericaval, superior mesentery artery, and/or celiac artery lymph nodes.

41 (41.0%) of the cases had distant metastasis (pm): distant metastasis.

Table 3: Summary of Scores of Immuno-markers in Gall bladder lesion cases

Scores	Mean ± SD	Median (IQR)	Min - Max
p53 Scores	2.89 ± 1.02	3.00 (2.00-4.00)	1.0 - 4.0
Ki67 Scores	2.87 ± 1.10	3.00 (2.00-4.00)	1.0 - 4.0
Egfr Scores	2.68 ± 1.02	3.00 (2.00-3.25)	1.0 - 4.0
Her2/Neu Scores	2.25 ± 0.58	2.00 (2.00-3.00)	1.0 - 3.0
Cyclin D1 Scores	2.51 ± 1.13	2.00 (2.00-4.00)	1.0 - 4.0
E-Cadherin Scores	1.89 ± 1.12	2.00 (1.00-3.00)	0.0 - 4.0

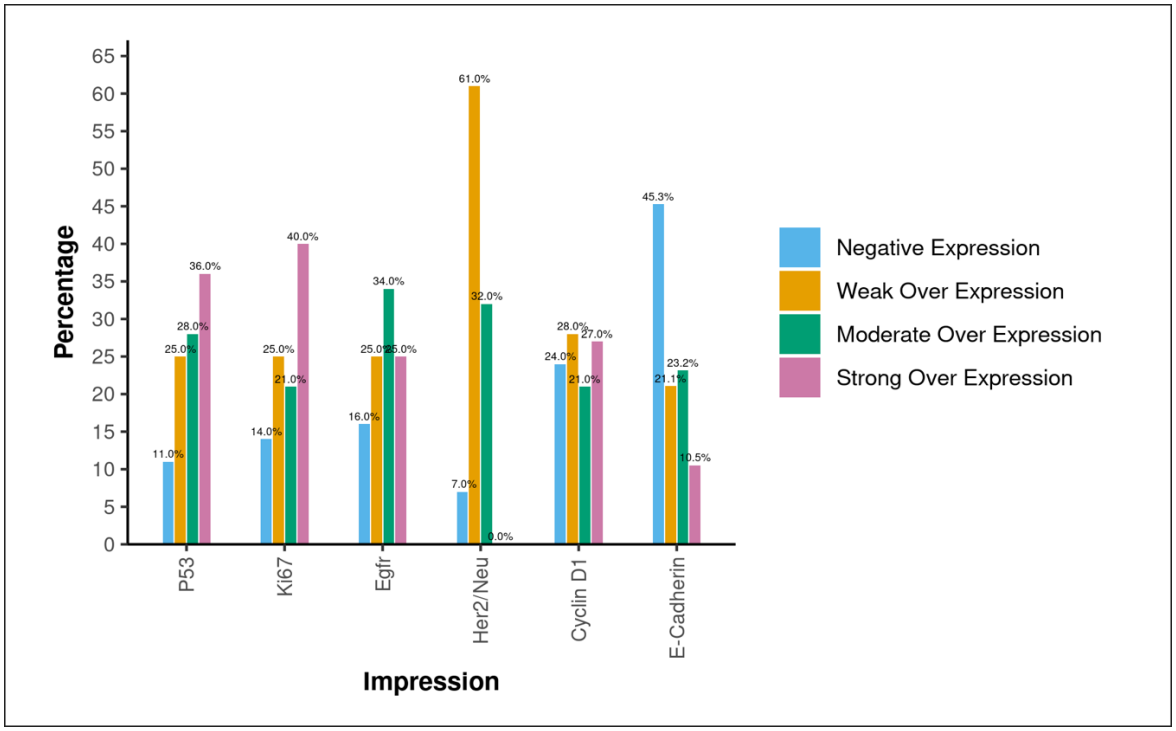


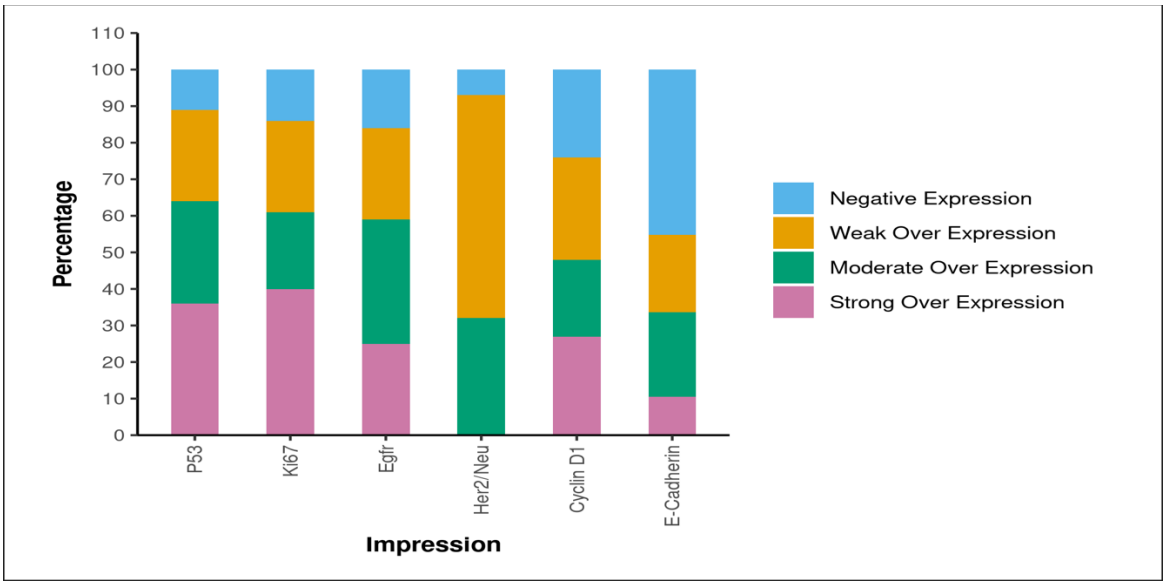


The mean score of immuno-markers in Gall bladder lesion cases is as follows:the mean p53 scores was 2.89 ± 1.02, mean Ki67 scores was 2.87 ± 1.10, mean EGFR scores was 2.68 ± 1.02, mean Her2/Neu scores was 2.25 ± 0.58, mean Cyclin D1 scores was 2.51 ± 1.13 and mean E-Cadherin scores was 1.89 ± 1.12 respectively.

Table 4: Summary of Immuno-markers expression in Gall Bladder lesion cases

Impression	Negative Expression	Weak Over Expression	Moderate Over Expression	Strong Over Expression
P53	11 (11.0%)	25 (25.0%)	28 (28.0%)	36 (36.0%)
Ki67	14 (14.0%)	25 (25.0%)	21 (21.0%)	40 (40.0%)
Egfr	16 (16.0%)	25 (25.0%)	34 (34.0%)	25 (25.0%)
Her2/Neu	7 (7.0%)	61 (61.0%)	32 (32.0%)	0 (0.0%)
Cyclin D1	24 (24.0%)	28 (28.0%)	21 (21.0%)	27 (27.0%)
E-Cadherin	43 (45.3%)	20 (21.1%)	22 (23.2%)	10 (10.5%)





Summary of Immuno-markers expression in Gall Bladder lesion cases was as follows:

Moderate over expression for P53 was seen in 36 (36.0%) of the cases. 21 (21.0%) of the cases had Ki67 moderate over expression and 40 (40.0%) of the cases had strong over expression of Ki67. 34 (34.0%) of the cases had EGFR moderate over expression and 25 (25.0%) of the cases had EGFR strong over expression respectively.61 (61.0%) of the cases had Her2/Neu had weak over expression and 32 (32.0%) of the cases had Her2/Neu had moderate over expression. 28 (28.0%) of the cases had weak over expression of Cyclin D1 and 27 (27.0%) of the cases had Cyclin D1 strong over expression.43 (45.3%) of the cases had E-Cadherin Negative Expression and 22 (23.2%) of the cases had Moderate Over Expression of E-Cadherin.

Discussions:

In order to improve treatment efficiency in Gall Bladder carcinoma (GBC), there is a need for accurate and early diagnosis followed by targeted therapy options. Identifying specific prognostic and diagnostic biomarkers and prospective patients for targeted therapies has become vital in treating GBC. Recent understanding of Gallbladder carcinogenesis has opened up avenues to develop useful immune-markers that could help identify disease progression and help patients receive personalised and targeted treatment.

In this study we have tried to explore the role of some immune-markers which may be useful in diagnosis of GBC precursor's lesions and detecting gall bladder carcinoma cases at early stage. Several categories of such immune-markers that have been studied by us are as follows: tumour suppressors like p53, E-Cadherin ,K67,EGFR,Cyclin D1,Her2/Neu etc.

P53

The TP53 gene is located in the small arm of Chromosome 17 is translated into a 53-kDa nuclear phosphoprotein tumour suppressor protein called p53 [6]. Mutations of the TP53 gene is one of the commonly observed genetic abnormalities in most human cancers [7]. During carcinogenesis, the tumour suppressor function of p53 is lost frequently due to point mutation, missense mutation or deletion of allele. Total loss of p53 protein is observed in cases of nonsense mutation or methylation of the p53 gene [6-7].

In GBC, the role of aberrant expression of p53 has been widely investigated. Wang *et al*, based on their study suggested that p53 overexpression significantly varied between carcinomas, precursor lesions and normal epithelia. They postulated that since adenomas did not express the same p53 abnormalities found in GBC, P53 overexpression might not have a significant role in the adenoma to carcinoma pathway [8]. While, p53 overexpression frequency is generally higher in GBC cases, it cannot be correlated to patient survival or tumour differentiation [9]. While some studies indicate that overexpression of p53 could be an early event in the carcinogenesis of well-differentiated adenocarcinomas of the gall-, others define p53 overexpression as a late event in the carcinogenesis process due to increased levels observed in cases from ominously progressed GBC [10]. P53 levels has been suggested as a means to predict recurrence of malignancy after surgical resection by immunostaining of tissue sample [11]. Kaur *et al*, linked p53 overexpression inversely to the grade of tumour and suggested p53 might not play a role in the metastasis of GBC [12].

Ambiguity reported regarding the role of p53, can be resolved with finding out optimized scoring protocol. More over understanding the individual role of p53 overexpression, loss and complete absence of the same can bring about a greater understanding about their impact in carcinogenesis. With more standardized studies, P53 could become a valuable tool in early diagnosis and treatment of GBC.

The following variables were significantly associated ($p<0.05$) with the variable 'p53 Scores' in our study: , Age, Site, Lesion, Neoplastic Lesion, Non-Neoplastic Lesion, Histological Grade, Pathologic Staging (pTNM), Regional Lymph Nodes (pN), Distant Metastasis (pM),

There was a significant difference between the 7 groups in terms of p53 Scores ($\chi2 = 12.802$, $p = 0.046$), with the median p53 Scores being highest in the Age: 41- 50 Years group.
There was a significant difference between the 5 groups in terms of p53 Scores ($\chi2 = 22.928$, $p = <0.001$), with the median p53 Scores being highest in the gall bladder site: fundus .

There was a significant difference between the 20 groups in terms of p53 Scores ($\chi2 = 73.330$, $p = <0.001$), with the mean p53 Scores being highest in the Lesion: Papillary Adenocarcinoma group. There was a significant difference between the 20 groups in terms of p53 Scores ($\chi2 = 73.330$, $p = <0.001$), with the mean p53 Scores being highest in the Lesion: Undifferentiated Carcinoma group. There was a significant difference between the 20 groups in terms of p53 Scores ($\chi2 = 73.330$, $p = <0.001$), with the mean p53 Scores being highest in the Lesion: Carcinoma, Not Otherwise Specified group.

. There was a significant difference between the 13 groups in terms of p53 Scores ($\chi2 = 23.174$, $p = 0.026$), with the mean p53 Scores being highest in the Non-Neoplastic Lesion: Eosinophilic Cholecystitis group. There was a significant difference between the 13 groups in terms of p53 Scores ($\chi2 = 23.174$, $p = 0.026$), with the mean p53 Scores being highest in the Non-Neoplastic Lesion: Intestinal Metaplasia group.

KI-67

The KI-67 antigen is a nuclear protein with two isoforms of 345KDa and 395KDa [13]. Healthy cells in resting phase do not exhibit KI-67 and its concentration actively changes throughout the cell cycle [14]. It helps maintain the compactness of the heterochromatin during the active phase of the cell cycle [15]. Ki-67 is overexpressed in actively proliferating tumour cells and hence it is used as an indicator of cancer. Detecting Ki-67 expression by measuring KI-67 LI through nuclear staining using immunohistochemistry is a widely used prognostic tool. Ki-67 Labelling Index [LI] for any given sample is the percentage of Ki-67 antigen-positive cells [16]. Higher Ki-67 LI, that is observed in cases of poorly differentiated tumours might be due to their tendency to rapidly proliferate [13]. Ki-67 LI can be an important tool to predict the aggressiveness of GBC. MIB, a monoclonal antibody with a higher affinity to Ki-67 has been lately used to evaluate its levels [14]. Higher MIB1 LI is linked to poorly differentiated tumours, lymph node metastasis and poor survival rate [13].

Ki-67 expression in GBC is found to be higher than in normal and benign conditions of gallbladder. A minor correlation between Ki-67 expression to the age and gender patients has also been studied and Ki-67 expression has been found at a greater frequency among patients of < 40 years and women [16]. Ki-67 was used to demonstrate increased cellular proliferation in cells of invasive gallbladder carcinoma [17]. Higher Ki-67 levels in GBC patients have been further correlated with poorly differentiated tumours and lymph node metastasis [17]. Ki-67 LI increases with histological grade in GBC [18]. Ki-67 LI shows measurable differences between benign and malignant states of the gallbladder epithelia. This can be extrapolated to help identify the transformation potency of benign conditions towards malignancy. However, for effective clinical usage as a prognostic index, Ki-67 expression in GBC needs to be further critically evaluated and standardized.

The following variables were significantly associated ($p<0.05$) with the variable 'Ki67 Scores' in our study: , Type of Gall Bladder

Lesions, Site, Lesion, Neoplastic Lesion, Non-Neoplastic Lesion, Histological Grade, Pathologic Staging (pTNM), Regional Lymph Nodes (pN), Distant Metastasis (pM), p53 Scores, Egfr Scores, Cyclin D1 Scores, E-Cadherin Scores, P53, Ki67, Egfr, Cyclin D1, E-Cadherin

In our study there was no significant difference between the age groups in terms of Ki67 Scores ($\chi2 = 9.407$, $p = 0.152$).

There was a significant difference between the 5 groups in terms of Ki67 Scores ($\chi2 = 20.076$, $p = <0.001$), with the median Ki67 Scores being highest in the gall bladder Site: fundus.

There was a significant difference between the 2 groups in terms of Ki67 Scores ($W = 1544.000$, $p = 0.034$), with the median Ki67 Scores being highest in the Type of Gall Bladder Lesions: Non-Neoplastic group of gall bladder lesions.

There was a significant difference between the 13 groups in terms of Ki67 Scores ($\chi2 = 24.903$, $p = 0.015$), with the median Ki67 Scores being highest in the Non-Neoplastic Lesion: Eosinophilic Cholecystitis group.

There was a significant difference between the 20 groups in terms of Ki67 Scores ($\chi2 = 65.877$, $p = <0.001$), with the mean Ki67 Scores being highest in the mucinous Adenocarcinoma cases.

Cyclin D1

Cyclin D1 coded by the CCND1 gene is a 295 amino acid protein. Significant levels of Cycling D1 overexpression has been observed in several cancers. Cyclin D1 overexpression can be a result of gene amplification, chromosomal rearrangement or disruption to the protein degradation [19].

Hui *et al* observed that higher mortality rates were correlated to the amplification of CCND1 gene in patients. They suggested that, CCND1 amplification and Cyclin D1 protein overexpression can be considered as independent events in carcinogenesis [20].

Hui *et al* proposed deregulation of Cyclin D1as an early-stage event. In their study if GBC samples, they observed Cyclin D1 overexpression in adenomas at a higher frequency in comparison to carcinomas and suggested that Cyclin D1 might play a role in the transformation of adenomas into cancer [20]. However, another study reported that there is no significant difference in the expression levels of Cyclin D1 between carcinomas and adenomas [21]. Higher cyclin D1 levels among poorly differentiated tumours and distant metastasis of GBC has been reported [26].

Significant difference in the expression levels of Cyclin D1 in benign conditions of the gallbladder has been observed. Benign conditions of the gallbladder showed lower or negative Cyclin D1 overexpression. Ma *et al* observed that chronic cholecystitis samples showed lower Cyclin D1 when compared to adenomas and adenocarcinomas of the gallbladder [21]. Nuclear and cytoplasmic staining of cyclin D1 at considerably higher levels were also observed in adenomas and low-grade dysplasia of the gall bladder epithelium [22].

These variations might help significantly in the early identification and treatment of GBC. Studying Cyclin D1 might further help in understanding the metastatic ability of GBC and the adenoma to carcinoma pathway.

The following variables were significantly associated ($p<0.05$) with the variable 'Cyclin D1 Scores' in our study: Type of Gall Bladder Lesions, Site, Lesion, Histological Grade, Pathologic Staging (pTNM), Regional Lymph Nodes (pN), Distant Metastasis (pM).

There was a significant difference between the 2 groups in terms of Cyclin D1 Scores ($W = 1537.000$, $p = 0.041$), with the median Cyclin

D1 Scores being highest in the Type of Gall Bladder Lesions: Non-Neoplastic group.

There was a significant difference between the 5 groups in terms of Cyclin D1 Scores ($\chi 2$ = 23.426, p = <0.001), with the median Cyclin D1 Scores being highest in the gall bladder site: neck of the gall bladder.

There was a significant difference between the 20 groups in terms of Cyclin D1 Scores ($\chi 2$ = 65.310, p = <0.001), with the mean Cyclin D1 Scores being highest in the gall bladder lesion: Adenosquamous Carcinoma group.

EGFR

Epidermal Growth Factor Receptor (EGFR) is a part of the ErBb family and is a transmembrane receptor tyrosine kinase [23]. It is made up of a single polypeptide chain consisting of 1186 amino acids and is usually located in the cell membrane of healthy cells. Higher levels of EGFR brought about by gene mutation or amplification can result in an abnormal increase in cellular proliferation [24]. Mutations of the EGFR gene are generally classified as 1. heterozygous which is the amplification of wild-type sequence on the second allele, or 2. homozygous/ hemizygous which is the amplification of the mutated sequence alone [25]. Increased EGFR levels have been reported in both benign and malignant conditions of the gallbladder. Gene mutation, amplification or translational upregulation of EGFR could possibly contribute towards higher-than-normal levels of EGFR observed in tumour cells. Overexpression of EGFR has been also observed in poorly differentiated tumours that show resistance to conventional treatment options and therapies [23-26].

In the case of gallbladder, studies have reported very low or negative immunoreactivity in benign samples of chronic cholecystitis and dysplasia [27,28]. In case of Cholelithiasis, significant number of samples tested showed increased EGFR levels. However, the reported levels were lower in comparison to that observed in GBC samples. Poorly differentiated tumours in GBC showed higher levels of EGFR than well differentiated ones [55]. Histological differentiation of GBC is inversely correlated to EGFR overexpression. Kaufman *et al* suggested the assumption that as poorly differentiated tumours behave more aggressively, EGFR expression levels may evince the extent of aggressiveness of GBC. Higher EGFR levels in patients could be correlated with shorter survival times [49]. Elevated EGFR levels in GBC can be considered as an independent prognostic variable among patients and can be an indication of adverse prognosis [29,30].

In another study, EGFR was overexpressed in 16% of GBC samples but was not observed in extrahepatic and intrahepatic bile duct cancer samples or normal epithelia or cholecystitis. Further they were unable to find any significant correlation between EGFR and Her -2 expressions in GBC [31].

EGFR protein overexpression studied through IHC was generally scored as follows: **0**= no staining observed; **1+**= faint membrane staining in > 1% of cancer cells in part of the cell membrane; **2+**= weak to moderate complete membrane staining in over 1% of cancer cells; Score 3+= intense complete membrane staining in over 1% of tumour cells [28,30]. Though, EGFR overexpression in GBC has been established through several studies, a standardized approach to quantifying the same is yet to be developed.

The following variables were significantly associated ($p<0.05$) with the variable 'EGFR Scores': , Site, Lesion, Neoplastic Lesion, Non-Neoplastic Lesion, Histological Grade, Pathologic Staging (pTNM), Regional Lymph Nodes (pN), Distant Metastasis (pM).

There was a significant difference between the 5 groups in terms of EGFR Scores ($\chi 2$ = 24.258, p = <0.001), with the mean EGFR Scores being highest in the gall bladder site: neck of the gall bladder.

There was a significant difference between the 20 groups in terms of EGFR Scores ($\chi 2$ = 83.878, p = <0.001), with the mean EGFR Scores being highest in the Lesion: Papillary Adenocarcinoma group, Undifferentiated Carcinoma group respectively and with the mean EGFR Scores being highest in the Lesion: Carcinoma, Not Otherwise Specified group.

There was a significant difference between the 13 groups in terms of EGFR Scores ($\chi 2$ = 29.814, p = 0.003), with the median EGFR Scores being highest in the Non-Neoplastic Lesion of the gall bladder: Pyloric Gland Metaplasia group.

E-Cadherin

E-cadherin is a 120KDa glycoprotein that functions to establish and maintain the Adherens Junctions (AJ) between cells through calcium mediation [31,32]. It is a tumour suppressor protein and also is one of the most studied biomarkers of human cancer. Loss of E-cadherin results in higher metastatic potential and apoptosis resistance in tumour cells [33]. Studies show that the concentration of membranous and cytoplasmic E-cadherin levels exhibit progressive reduction from normal gallbladder epithelia to inflamed tissue to GBC. Reduced E-cadherin levels on the cytoplasmic membrane was observed in undifferentiated tumours in GBC patients [34]. Decreased E-cadherin expression has been associated with metastases, extent of wall invasion in GBC apart from increase in the proportion of undifferentiated tumours [35]. E-cadherin inhibition also offers a potential therapeutic value as Na *et al* demonstrated that activating E-cadherin using mAbs can inhibit metastasis at different stages through various pathways [36]. The reducing E-cadherin levels could be tracked and exploited to identify the malignancy potential of benign conditions and stage GBC with further studies.

The following variables were significantly associated (p<0.05) with the variable 'E-Cadherin Scores' in our study : Neoplastic gall bladder lesions

There was a significant difference between the 20 groups in terms of E-Cadherin Scores ($\chi 2$ = 55.951, p = <0.001), with the median E-Cadherin Scores being highest in the Lesion: Adenocarcinoma group.

HER2

HER2 is another transmembrane tyrosine kinase receptor protein whose overexpression has been detected and correlated to the progression of several human cancers [37]. Overexpression of HER2 in GBC has been demonstrated in several studies [38,39] but the frequency of overexpression and gene amplification has been found to be lower than other markers. HER2 protein overexpression in relation to gene amplification is yet to be established in GBC [38]. Studies also suggest that HER2 protein overexpression in GBC could be more often due to gene deregulation rather than amplification [17]. HER2 levels reported across different studies conducted on GBC samples exhibit a large variation. These reported variations in HER2 expression have been attributed to the choice of different assays and scoring systems with varying specificity and sensitivity towards GBC [39]. The most commonly used scoring systems in these studies are based on the criteria used for gastric cancer [40] and breast cancer [38-41] along with the Hercep test scoring system [28-42]. Though, Toledo *et al* argued that HER2 immunoreactivity observed in basolateral cell membrane domain of metaplastic gallbladder epithelium in GBC could not be compared to that scored by Hercep Test as it is based on the immunoreactivity observed in invasive breast cancer cells that are neoplastic non-polarized cells, Kawamato *et al* suggested that Hercep test could be used as an acceptable predictor to determine FISH positivity of HER2 gene amplification [28-43]. It has also been suggested that HER2 overexpression could be more prevalent in precursor lesions than

carcinoma *in situ* and metastasis [30,44-45]. If this is so, then investigations specifically aimed at understanding the role and concentration of HER-2 levels in benign and precancerous lesions might help reveal its potential in becoming an invaluable prognostic tool in the early identification of malignant transformation.

The following variables were significantly associated ($p<0.05$) with the variable 'Her2/Neu' in our study: , Age, Type of Gall Bladder Lesions, Neoplastic Lesion, Non-Neoplastic gall bladder Lesions .

There was a significant difference between the 7 groups in terms of Her2/Neu Scores ($\chi 2 = 23.444$, $p = 0.001$), with the median Her2/Neu Scores being highest in the Neoplastic Lesion: Papillary Adenocarcinoma group.

Ki67 Scores, Cyclin D1 Scores, Her2/Neu Scores significantly predicted Type of neoplastic Gall Bladder Lesions with the trend being as follows

Trends:.

Best parameter in terms of sensitivity: EGFR Scores.

Best parameter in terms of specificity: Cyclin D1 Scores.

Best parameter in terms of positive predictive value: Cyclin D1 Scores.

Best parameter in terms of negative predictive value: EGFR Scores.

Best parameter in terms of diagnostic accuracy: Ki67 Scores.

Conclusions:

Gallbladder Carcinoma, though rare, is a deadly form of cancer with limited treatment options. Identifying prognostic markers specific to GBC can change the current course of clinical approach making way for better and targeted prognostic and patient-oriented treatment practices when conventional treatment options may not be viable. These molecular based diagnostic and prognostic tools may bring about a better understanding of the disease and thus warrant further research and standardization studies that will lead to their clinical applicability. Studying the above discussed biomarkers on molecular and analytical levels about their role in benign diseases, benign to malignant transformation, primary tumours and metastasis will help open up avenues for a better prognostic approach to tackle GBC and increase overall survival time in patients.

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