

Clinicopathological And Prognostic Significance Of Immuno-Expression Of Cyclin D1, E-Cadherin, EGFR, HER- 2, Ki67, And P53 In Gall Bladder Cancer And Its Precursor Lesions-A Review

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Introduction:

Gallbladder carcinoma (GBC) is an aggressive and common cancer of the biliary tract. It develops on 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 rarely presents a distinct set of clinical symptoms, delayed diagnosis contributes heavily towards the negative prognosis widely associated with the disease [2]. The molecular mechanisms and underlying changes that bring about carcinogenesis of the gallbladder epithelia, though widely studied have not been fully understood. Chronic inflammation [3], dysplasia [4] and adenoma [5] among other factors have been observed to increase the risk of developing GBC among other risk factors. 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 molecules 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 [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. Several categories of markers have been studied to date and include tumour markers such as CA125, CA19-9, CEA, inflammatory markers like CRP, tumour suppressors like p53, E-cadherin etc. In this review we hope to explore the future prospects of some of these markers based on available literature.

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 [8]. Mutations of the TP53 gene is one of the commonly observed genetic abnormalities in most human cancers [9]. 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 [8].

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 [10]. While, p53 overexpression frequency is generally higher in GBC samples, it cannot be correlated to patient survival or tumour differentiation [11]. However, in another study p53 overexpression in GBC was found to influence poor prognosis and adverse clinical outcome in patients [12]. While some studies indicate that overexpression of p53 could be an early event in the carcinogenesis of well-differentiated adenocarcinomas of the gall-bladder [13], others define p53 overexpression as a late event in the carcinogenesis process due to increased levels observed in samples from significantly progressed GBC [14,15]. p53 levels has been suggested as a means to predict recurrence of malignancy after surgical resection by immunostaining of remnant tissue sample [16]. 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 [17].

Ambiguity reported regarding the role of p53, can be resolved with optimized and standardized assays and scoring protocol. More over understanding the individual role of p53 overexpression, loss of wildtype protein and complete absence of the same can bring about a greater understanding about their impact in carcinogenesis. This data can be extrapolated further for its prognostic potential as its overexpression in GBC has been linked to tumour grade, differentiation and outcome. With more standardized studies, P53 could become a valuable tool in early diagnosis and treatment of GBC.

IL 17A

Interleukin 17 A, commonly abbreviated as IL 17 A or IL17 is secreted by TH 17 cells. It plays a major role in the immune response against pathogens, allergies and autoimmune disorders [18]. Its role in various cancers is being widely studied. In GBC, it is thought to aid cell proliferation and have a pro-metastatic role.

Patil et al, established that IL 17 increased cellular proliferation in GBC and reported that IL17 producing cells $\gamma\delta 17$ and Th17 as predictive markers in GBC. Future treatment options could target $\gamma\delta 17$'s movement to the tumour site and inhibition of IL 17 function [19]. Chen et al studied the role of IL 17 in invasive nature of GBC. They established that IL 17 played a pro-invasive role in GBC. IL 17 aided EMT by increasing the activity of ERK-NF- κ B signaling pathway in which, vimentin and slug were upregulated and E-cadherin was downregulated [20]. Loss of E-cadherin and increased expression of vimentin and slug contributes to the invasive nature of GBC [20,21]. IL 17 plays an important role in defending the body against harmful pathogens. Hence, the complete implications and risks involved in the clinical application for prognosis and treatment methods including inhibition of IL-17 should be carefully studied [18].

KI-67

The KI-67 antigen is a nuclear protein with two isoforms of 345KDa and 395KDa [22]. Healthy cells in resting phase do not exhibit KI-67 and its concentration actively changes throughout the cell cycle [23]. It helps maintain the compactness of the heterochromatin during the active phase of the cell cycle [24]. 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 [25]. Higher Ki-67 LI, that is observed in samples of poorly differentiated tumours might be due to their tendency to rapidly proliferate [22,26]. 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 [23,27]. Higher MIB1 LI is linked to poorly differentiated tumours, lymph node metastasis and poor survival rate [22,28].

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 [26,29]. Ki-67 was used to demonstrate increased cellular proliferation in cells of invasive gallbladder carcinoma [30]. Higher Ki-67 levels in GBC patients have been further correlated with poorly differentiated tumours and lymph node metastasis [27]. Ki-67 LI increases with histological grade in GBC [31]. 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.

CEA

Carcinoembryonic antigen is one of the traditionally used tumour markers for gallbladder cancer [2]. Serum CEA can be used to differentiate GBC from benign gallstones [32]. GBC patient samples that were positive for cytoplasmic CEA and stromal CEA frequently showed Lymph node metastasis [33]. Also, CEA- dNLR (derived neutrophil-to-lymphocyte) ratio is an independent prognostic factor in GBC can be used to predict the overall survival [34]. increased CEA levels have been correlated with metastasis in GBC patients [35]. CEA, might not be clinically effective as a standalone prognostic marker and however, its specificity can be increased in combination with other biomarkers.

CA19-9

Carbohydrate Antigen 19-9 (CA19-9) serum level has been identified as an independent prognostic factor among GBC patients and can be used as an indicator to determine the need for adjuvant therapies when conventional therapies might not be as effective [36]. CA19-9 can also be used to differentiate GBC from benign conditions of the gallbladder [32]. Though, CA19-9 exhibited the highest sensitivity it cannot be used as a standalone marker to identify GBC due to its low specificity [37].

CA125

CA125 (Cancer Antigen 125) is another tumour marker that is widely used in GBC diagnosis [2]. At the cut off value of 11 U/ml, CA 125 was able to differentiate GBC from benign conditions of the gallbladder with a sensitivity of 64% and specificity of 90%. Increased CA 125 could be used to differentiate GBC from (glycogen storage disease) GSD [38]. CA 125 with a cut off value of 92.19U/ml was found to have a sensitivity of 100% and specificity of 94.5% [39]. Though, CA125 could play a significant role in diagnosis of GBC its prognostic potency remains to be established.

CRP

C-reactive protein (CRP) is a serum protein originating hepatically. Increased CRP levels are directly correlated to the inflammatory response as a result of disease or injury. As such it is called as prototypic acute phase reactant [40].

Significantly elevated CRP levels can be associated with cholecystitis and gallbladder cancer as both conditions show considerable inflammation. Chronic inflammation of the gallbladder brought about by gallstones or infections can result in higher serum CRP levels, thereby increasing GBC risk, while inflammation resulting from carcinoma contributes to the higher CRP levels observed in GBC patients [41]

Kim et al, conducted a comparative study on the CRP level between acute cholecystitis and gallbladder cancer patients. While they found increased CRP levels in both cases, the level was noticeably higher among patients with acute cholecystitis than that of GBC patients. CRP levels have been associated with poor prognosis in GBC patients [42].

CRP level in serum is non-specific and can be used in conjunction with other specific tools to assess progress of GBC or the possibility of its recurrence.

Cyclin D1

Cyclin D1 coded by the CCND1 gene is a 295 amino acid protein. Significant levels of Cyclin 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 [43]. Cyclin D1 overexpression can also be brought about by alterations of the associated signaling intermediates, including the RAS–MEK–ERK and PI3K pathways [44, 45].

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 [46].

Hui et al proposed deregulation of Cyclin D1 as an early-stage event. In their study of 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 [46]. However, another study reported that there is no significant difference in the expression levels of Cyclin D1 between carcinomas and adenomas [47]. Doval et al reported higher cyclin D1 levels among poorly differentiated tumours and distant metastasis of GBC [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 [47]. 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 [48].

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.

EGFR

Epidermal Growth Factor Receptor (EGFR) is a part of the ErbB family and is a transmembrane receptor tyrosine kinase [49]. 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 [50]. 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 [51]. 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 [49,51,52].

In the case of gallbladder, studies have reported very low or negative immunoreactivity in benign samples of chronic cholecystitis and dysplasia [53,54]. 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 [55,56].

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 [59].

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 [54,56]. Though, EGFR overexpression in GBC has been established through several studies, a standardized approach to quantifying the same is yet to be developed.

E-Cadherin

E-cadherin is a 120KDa glycoprotein that functions to establish and maintain the Adherens Junctions (AJ) between cells through calcium mediation [57,58]. 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 [59]. 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 [60]. Decreased E-cadherin expression has been associated with metastases, extent of wall invasion in GBC apart from increase in the proportion of undifferentiated tumours [61]. 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 [62]. The reducing E-cadherin levels could be tracked and exploited to identify the malignancy potential of benign conditions and stage GBC with further studies.

HER2

HER2 is another transmembrane tyrosine kinase receptor protein whose overexpression has been detected and correlated to the progression of several human cancers [63,64]. Overexpression of HER2 in GBC has been demonstrated in several studies [64,65,66] 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 [65]. Studies also suggest that HER2 protein overexpression in GBC could be more often due to gene deregulation rather than amplification [30,67]. 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 [66]. The most commonly used scoring systems in these studies are based on the criteria used for gastric cancer [68] and breast cancer [66] along with the Hercep test scoring system [54]. 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, Kawamoto et al suggested that Hercep test could be used as an acceptable predictor to determine FISH positivity of HER2 gene amplification [54]. It has also been suggested that HER2 overexpression could be more prevalent in precursor lesions than carcinoma in situ and metastasis [30]. 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.

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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