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REVIEW

Pathology of Gallbladder Carcinoma: Current Understanding and New Perspectives

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Abstract Gallbladder carcinoma is a rare and highly lethal malignancy. It stands out from amongst the other GI tract malignancies for its unique epidemiological profile, proclivity for female gender, definitional ambiguities, ability to escape early diagnosis, and absence of effective treatment. Pathobiology of gallbladder carcinoma continues to remain poorly understood. Recently, better characterization of the precursor lesions and elucidation of underlying molecular pathways has enhanced our understanding of gallbladder tumorigenesis. Proposal of a unified terminology and evolving consensus in classifying gallbladder pre-invasive neoplasia offers hope of better assimilation of rare data from diverse parts of the world. Identifying biomarkers and cancer specific cellular targets that will pave the way for novel therapeutic approaches for gallbladder carcinoma is urgently needed. In this review we delve into the epidemiologic, genetic and pathologic characteristics of this enigmatic disease with a special focus on the recent advancements in the field of gallbladder pathology.

Keywords Gallbladder carcinoma · Epidemiology · Pathways · Terminology · Precancerous lesions · Histologic subtypes · Molecular pathology

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Introduction

Gallbladder carcinoma (GBC) is a notoriously lethal malignancy with dismal outcome. Although rare as such, GBC is the most frequent amongst all biliary tract cancers [1]. Typically, by the time a carcinoma of the gall bladder (GB) is discovered, the cancer has surpassed its chances of cure. Not infrequently, the diagnosis comes as an unpleasant surprise following a cholecystectomy performed for symptoms of chronic cholecystitis. Much of this is imputable to its remarkable ability to remain unsuspected during many levels of evaluation; vague symptomatology, nonspecific radiology and deceptive gross appearances often project an innocuous picture on clinical, imaging and pathological examination, allowing it to escape early detection. As a result, prognosis of gallbladder carcinoma (GBC) patients has remained abysmal, with less than 5 % 5 year survival [1]. In spite of huge advancements in understanding and treatment of other gastrointestinal (GI) malignancies, GBC treatment and outcome has hardly witnessed any progress. Its rarity and prevalence in far-flung pockets of the world as well as existence of a plethora of ambiguous nomenclature for overlapping pathological entities have allowed gaping lacunae in our knowledge of GBC tumorigenesis to persist. There is an urgent need to understand the mechanisms of GB carcinogenesis and to unravel a clear picture of its genetic landscape, and then to translate that information into robust classification systems based on unified terminologies. This can prompt a much needed convergence of rare information from remote corners of the world into a compendium of meaningful data that may yield promising biomarkers, novel treatment breakthroughs and eventually ameliorate outcome of this deadly disease. In this review, we reflect upon the epidemiological, genetic and pathologic characteristics that make GBC distinctive and focus upon the advancements made in the arena of gallbladder pathology in the recent times.

Epidemiology

For years, carcinoma of the gallbladder has enamored epidemiologists. A proclivity for a distinctive demographic profile and a cavernous disparity in geographical endemicity bestows GBC a unique epidemiological signature. Gallbladder carcinoma's extremely low incidence in most of the developed world contrasts starkly with its inordinately high prevalence in some of the developing countries. Moreover, differences exist amongst diverse ethnic and racial populations inhabiting the same regions. In the United States, GBC accounts for 0.5 % of all GI malignancies (the fifth most common cancer of the GI tract) with a low incidence rate of 1–2.5/100,000 people [1, 2]. Similar rates are observed in Canada and Northern Europe (<2/100,000) [3]. On the other hand, highest incidence rates are reported from Native American and South American populations, especially Chile's Mapuche Indians (23/100,000), Northern India (21.5/100,000), Pakistan (11.3/100,000), South America (mortality rates of 15.5/100,000) and Eastern Europe (14/100,000 in Poland) [3, 4]. Israel (5/100,000) and Japan (7/100,000) also show escalated rates while a rising trend has been witnessed in China (doubling of rates in Shanghai over the last 20 years) [3, 5]. Highest mortality rates, exceeding those of breast and cervix, are reported in Chilean women [6, 7]. Interestingly, even within geographical precincts, a schismatic dichotomy in incidence rates of GBC exists. While one of the highest incidence rates of the world are reported from North India (10.1–21.5/100,000 amongst women in Delhi) [1, 3], the disease is hardly prevalent in South India (incidence in Chennai, Trivandrum, Bangalore, and Mumbai is 0.9-2/100,000) [1]. In the USA, GBC is more commonly seen in Native American Indians, and Hispanics [8]. The strong geographical, ethnic and racial disposition of GBC occurrence underscores an intricate and complex interplay of genetic and environmental factors active in its etiopathogenesis.

Risk Factors and Etiopathogenesis

The exact etiology of GBC remains obscure; however risk factors that augment the odds of developing this malignancy are now well recognized (Table 1). These also offer insights, albeit in some measure, into the underlying pathogenetic basis for its geographical and ethnic disposition. Two key risk factors associated with GBC are: (a) chronic inflammation, from any source, that evokes an incessant release of mediators of inflammation, active oxygen radicals, toxins and metabolites which are potentially mutagenic and impact cell cycle regulation [9] and (b) an underlying genetic susceptibility to GBC attained from inheriting an altered gene pool that encodes for protein machinery engaged in handling bile transport and metabolism.

Table 1 Risk factors of gallbladder carcinoma

Factor	Relative risk (RR)/ Odds ratio (OR) / Association (A)	[References]
Gallstones	4.9 (RR)	[3]
Stones 2.0–2.9 cm	2.4 (RR)	[12, 13]
Stones>3 cm	9.2–10.1 (RR)	[12, 13]
Bile infection		
Salmonella sp.	12.7 (RR)	[21, 24]
Helicobactor sp.	4.3 (RR)	[3]
Environmental carcinogens	–	
Porcelain gallbladder	8.0 (RR)	[31, 32]
APBJ	3–18 % (A)	[36–38]
Autoimmune syndromes		
PSC	14 % (A)	[40]
UC	10 % (A)	[41, 42]
Genetic polymorphisms		
APOB X(+) D haplotype	2.90 (OR)	[44]
CR 1 (GG genotype of A3650G RsaI and intron 27 HindIII)	1.99 (OR)	[48]
CC genotype and variant allele of CYP7A1 A(204)C	3.30 (OR)	[49]
LRPAP1 (D allele)	1.60 (OR)	[50]
CCR5+/Delta32 genotype	2.85 (OR)	[51]
CCR5 Delta32 allele	3.15 (OR)	[51]
Dietary factors	–	
Oestrogen	–	
Obesity (Body mass index>30 kg/m^2)	1.8 (men) and 2.1 (women) (RR)	[10]

APBJ Anomalous pancreatobiliary junction, *PSC* Primary sclerosing cholangitis, *UC* Ulcerative colitis, *APOB* Apolipoprotein B, *CR1* Complement receptor 1, *CYP7A1* Cholesterol 7-alpha hydroxylase, *LRPAP1* Low-density lipoprotein receptor-related protein associated protein, *CCR5* Chemokine receptors

Gall stones have a strong clinical association, albeit lacking in experimental data, to GBC: gallstones are present in approximately 65–90 % of GBC patients [1]; gallstones share common epidemiological and demographic distribution with GBC [10, 11]; and risk of GBC escalates with increasing stone size (≥3 cm have a risk of 4 % over 20 years [12, 13]). Higher risk of GBC development with larger stones possibly reflects the greater duration and intensity of epithelial irritation [13]. Further supportive evidence comes from findings of epithelial dysplasia, atypical hyperplasia, and carcinoma in situ (seen in 83, 13·5, and 3·5 % of patients, respectively), in the GBs removed for treatment of gallstones [14, 15]. In spite of a strong association, the role of gallstones appears more of a co-factor than a causal agent [16, 17]. The presence of stones alone is insufficient; gallstones remain asymptomatic in 66–77 % of the general population [18] and only 0.3–3 % patients with cholelithiasis develop GBC [19].

Bile Infection several studies have shown a strong association of chronic bacterial infection with GBC, particularly Salmonella infections in areas of high typhoid endemicity, such as India [9, 20–23], with a life time risk of developing GBC of 6 % (a 12-fold increased risk) [21, 24]. A study from North India detected chronic Salmonella carriage by 67 % patients of GBC compared to healthy controls (Odds ratio 22.8) [9]. Lodged in its hepatic niche, Salmonella is intermittently excreted into the GB, where bacterial enzymes (glucoronidases) breakdown bile acids and nitrates into carcinogenic secondary bile acids and nitroso compound, respectively [9, 25, 26]. Complying with its daily function of concentrating bile, the GB ends up bearing the brunt of this amplified mutagen contact. Moreover, bacteria themselves act as a nidus for gallstone formation. With the ensuing chronic inflammation resulting in fibrosis and impaired mobility of the GB, the protracted exposure aggravates the damage. Further, direct DNA damage from Salmonella's cytolethal distending toxin (CDT) and immune evasive properties leads to a recalcitrant infection that sets a perpetual cycle of injury in motion, eventually inducing neoplastic transformation [27]. Other bacterial pathogens, such as Helicobacter species (H bilis, H pullorum, H hepaticus, H pylori) are increasingly being implicated in GB carcinogenesis, however their direct role in etiology is yet unproven [3, 9]. GBC has been correlated to poor socioeconomic conditions, and bile infection, if not alone, appears to be a dominant trigger leading to GBC development in resource-poor countries [28].

Excessive exposure to chemicals and pollutants, such as heavy metals such as nickel and cadmium, pesticides, radiation and vinyl chloride and industrial pollutants (paper, automobile, shoe, textile, oil, rubber and metal fabricating factories) are associated with increased GBC risk [29]. Further evidence comes from animal studies that demonstrate development of GBC with exposure to nitrosamines, methylcholanthrene, and O-aminoazotoluence [30]. Improper disposal of industrial effluents and chemicals into natural resources is rampant in many developing countries due to poor waste management laws and practices, and lack of regulations on pollution.

Chronic inflammation from any cause can trigger deposition of calcium within the GB wall, a phenomenon that renders its wall hard, bluish and brittle and earns it the nomenclature of a **'porcelain gall bladder'**. It is a rarity**,** occurring in less than 1 % of all the GB specimens, with studies reporting 13–62 % association [31, 32] while other studies negating the association [33, 34]. Interestingly, rather than the diffusely calcified GBs, those with stippled calcification tend to develop GBC [32] and the latter therefore, reasonably justify a cholecystectomy [35].

Anomalous pancreatobiliary junction (APBJ) is a rare congenital malformation in which the junction of pancreatic duct and bile duct resides outside the duodenal wall instead of at the ampulla. Liberated from sphincteric regulation, this anomaly allows the pancreatic juices to regurgitate into the biliary passages and the GB. The consequent chronic inflammation is believed to trigger hyperplasia-metaplasia-dysplasia sequence that eventually culminates in GBC. APBJ is estimated to contribute to a heightened risk (3–18 %) for developing GBC [36–38]. This condition is particularly more prevalent amongst young Asians (especially Japanese populations) and is not linked to gallstones. This maljunction is also associated with a much higher incidence of KRAS mutations compared to the other GBC cases [39].

Autoimmune and hereditary syndromes, such as primary sclerosing cholangitis (PSC) and ulcerative colitis (UC) have been known to confer an elevated risk of biliary carcinoma. Dysplasia and adenocarcinoma is found in 37 and 14 % of the GBs from patients with PSC while upto 10 times increased risk in GBC development is observed in UC patients compared to general population [41, 42]. The duration of symptomatic colitis might play an active role in the development, further propelling the inflammation-driven malignancy theory for GBC [43].

Genetic Susceptibility high prevalence amongst racial and ethnic groups is a strong clue towards an underlying genetic participation in the GBC narrative. Gallstones and GBC not only display identical geographical propensity, both have an undeniable genetic component as well. Alterations or polymorphisms (that tend to summate in close ethnic populations) in a multitude of genes engaged in trans-hepatic cholesterol and environmental toxins' disposal and bile synthesis modulate the susceptibility towards developing a GBC. Best identified genes include, variants of apolipoprotein B (APOB) and biliary lipid transporters in the canalicular membrane—the ATP binding cassette (ABC) transporters [44]. These include transporters of cholesterol (ABCG5/8), bile salt export pump (ABCB11), and phospholipids and lecithin (ABCB4). Alterations/ polymorphisms in these genes (especially, the ABCG8 gene variant D19H) result in increased cholesterol secretion or reduced lecithin in bile, stone formation, and an extended stay in a dyskinetic GB [45–47]. Polymorphisms in various alleles related to cholesterol and biliary metabolism, such as, complement receptor 1 [48], cholesterol 7-alpha hydroxylase (CYP7A1) [49], lipoprotein receptor associated protein (LRPAP1) [50], CCR5+/Delta32 genotype [51], epidermal growth factor (EGF) and transforming growth factor beta (TGFB1) alleles [52] and interleukin-1 receptor (IL1) haplotypes [53], to name a few, have been implicated in enhanced susceptibility of developing GBC. This posits that an intricate collaboration of altered genes (or variants involved in regulation of bile homeostasis) with environmental triggers enhances risk of developing GBC.

Gender and habitudes have been linked to GBC development. For many years, the five f's: 'fair, fat, fertile female of forty' have been handed down generations in medical schools to describe a very prototypical patient of GBC. The age-old mnemonic continues to remind a long acknowledged relationship of GBC to female gender, parity and obesity. However, much of the association is now understood to be a result of predisposition to the common risk factors of gallstones. A study on estrogen and progesterone expression in GBC failed to find significant expression [54]. Dietary factors, such as mustard oil adulterated by sanguinarine, diethylnitrosamine and repeated frying in North Indian cooking, have been suggested as an etiological factor [55]. Calorie- and carbohydrate-rich diets are speculated to increase the risk of acquiring GBC while ample intake of fruits and vegetables with their rich antioxidant content confer protection; latter is [56, 57].

Gallbladder polyp is a term that has been used for a non-specific, clinically detectable polypoid mass irrespective of its pathological identity. Hence, it has included a range of non-neoplastic, metaplastic and neoplastic polypoid masses. Polyps>1 cm have been considered a risk factor for GBC development [58] and form a clinical indication for cholecystectomy.

Pathways of GB Carcinogenesis

Tumorigenesis is a multi-step process resulting from cumulative genetic and epigenetic alterations affecting oncogenes and tumor suppressor genes. Clinical, pathological and molecular data indicate that there are two models of GB carcinogenesis [59]:

1. Metaplasia- Dysplasia-Carcinoma sequence
2. Adenoma-carcinoma pathway

Metaplasia- Dysplasia-Carcinoma sequence is the dominant path pursued by GBC and is elicited by key pathogenic events triggered by gallstones and chronic inflammation. This pathway is identifiable by a sequence of morphological alterations wherein normal epithelium morphs into a metaplastic type, and progressively acquires increasing grades of dysplasia that culminate in carcinoma in-situ (CIS) and invasive carcinoma. These alterations are identified in more than 90 % of GBC [60] and offer vital clues to the temporal sequence of events in the progression of GBC. Symptomatic cholecystitis tends to appear at a median age of 40 years, dysplasia at 45 years, CIS at 55 years and invasive carcinoma at 60 years [61]. Hence, a high risk individual has a prospect of a GBC surfacing 20 or more years after onset of chronic cholecystitis, 15 years past developing dysplasia and 5 years following CIS [61]. This knowledge is extremely potent as it offers an opportunity for timely intervention to avert progression to cancer.

Adenoma-carcinoma pathway reflects a road less travelled by GBC. There is evidence that a small proportion of GBCs begin as mass-forming glandular proliferations, customarily referred to as adenomas. Acquisition of increasing cytologic and architectural dysplasia by this mass forming lesion and eventual invasive traits are the morphological imprints of GBC's journey through this route [62, 63]. Studies report a low incidence of gallbladder adenomas (0.14–1.1 % of cholecystectomies) [64, 65] and adenomatous remnants in the mucosa adjacent to early carcinomas (3–7 % of the cases) [66]. Dursan et al. credited approximately 10 % of the invasive carcinomas to the adenoma-carcinoma pathway [67]. Little is known about the temporal progression and the molecular genetics of these lesions.

Precancerous Lesions of Gallbladder Carcinoma

As is the norm with most GI malignancies, biliary tract cancers also pursue a stepwise tumor progression model in which the invasive tumor is preceded by a well defined and morphologically distinctive pre-invasive stage. Characterization of pre-invasive lesions has tremendous value as they not only furnish important links in the understanding of tumorigenesis, but also present an opportunity to develop screening tools for high risk populations and halt progression of cancer in its early stages. The gallbladder has been known to harbor a variety of premalignant lesions. Over the years, owing to the rarity of experience with these lesions and an indiscriminate usage of nomenclature by pathologists in different pockets of the world, a vast lexicon of confusing terminologies ('pyloric gland adenoma,' 'papillary adenoma,' 'tubulopapillary adenoma,' 'biliary adenoma,' 'intestinal adenoma,' 'transitional adenoma,' 'papillary neoplasm,' papillary carcinoma,' and 'intracystic papillary neoplasm') has evolved for overlapping morphological entities [62, 68–86]. This has resulted in widely varying frequency and outcomes for these lesions. As a result, a clear picture of pre-cancerous lesions of the GB has remained elusive.

In recent years, an explosive expansion in the understanding of pancreatic precursor lesions has revived an acute interest in their counterparts in the GB. It is now amply clear that analogous to pancreatic cancer; biliary malignancies are also preceded by two distinct types of pre-invasive intra-epithelial neoplasia; the major point of distinction between the two types being their ability to form a mass lesion. Simplistically put, the two types of intra-epithelial neoplasia include:

a) non-tumoral or flat type, and
b) tumoral or mass-forming type.

The former are akin to the pancreatic intraepithelial neoplasms (PanINs) and biliary intraepithelial neoplasms (BilINs) of bile ducts which, by definition, are microscopic lesions [87, 88]. The latter, on the other hand, are comparable to intraductal papillary mucinous neoplasms (IPMNs) and intraductal tubulopapillary neoplasms (ITPNs) of pancreas and intraductal papillary neoplasms of bile duct (IPNBs) of bile duct [89–92].

A) Flat dysplasia / biliary intraepithelial neoplasia of gallbladder

Conventional flat dysplasia, also referred to as biliary intraepithelial neoplasia (BilIN) of the GB, is the most common precursor of GBC. Evidence of BilINs as precursor lesions of GBC is largely based on indirect evidence. More than 80 % GBC reveal foci of CIS or epithelial dysplasia [14, 80, 93, 94]; incidence of dysplasia is high in areas of high GBC incidence; a positive correlation is seen between increasing degree of neoplastic transformation and the mean age of patients [95]. In the absence of invasive carcinoma, these are uncommon and found in 0.5–3.5 % of cholecystectomy specimens removed for gallstones [60, 96, 97]. High grade dysplasia has also been detected in patients with familial adenomatous polyposis (FAP) syndrome, APBJ reflux and PSC [60, 98–103]. Nearly always, BilINs are an incidental finding. As these are flat lesions capable of being completely missed on even macroscopic examination, it is impossible to suspect their existence clinically.

Pathologic Findings

Parallel to the criteria for PanINs, BilINs are microscopic lesions and a size cut-off of<1 cm is applied to serve as a mark of distinction from the tumoral intra-epithelial neoplasms (TINs). On gross examination, these lesions are remarkably indiscernible; their presence is occasionally hinted at by granularity or plaque-like changes in the mucosa [104]. Microscopically, a normal gallbladder is lined by a single layered columnar lining. When subjected to chronic irritation, the GB resorts to an adaptive transformation into an alternate metaplastic lining; the commonest metaplasia is pyloric, followed by intestinal and infrequently squamous. However, metaplastic change is devoid of any cyto-nuclear abnormalities or dysplasia. Dysplasia is morphologically characterized by a non mass-forming disorderly proliferation of atypical cells with architectural and cyto-nuclear abnormalities limited to the epithelium; the lining epithelium may assume a variety of patterns: flat, clinging/ denuding, micropapillary, papillary (<1 cm, by definition) and glandular [88, 105]. The lesions can adopt diverse types of epithelium (biliary, gastric, intestinal, oncocytic, squamous or signet ring) [60, 106]; a mixture of different epithelial types may co-exist in a given case. Little is known about the clinical relevance of the cellular differentiation; gastric and intestinal types appear to impart an aggressive biology while goblet cells perhaps reflect maturation and thereby imply an indolent progression [107].

Based on the increasing quantum of cyto-architectural complexity and dysplasia, BilINs are classified histologically into three grades: BilIN1 (low grade dysplasia), BilIN2 (intermediate grade) and BilIN3 (high grade dysplasia) (Fig. 1). Notably, the morphological descriptions and criteria for grading GB dysplasia have been harvested from the pancreas and other GI sites and extended to biliary neoplasms [88]. While there is agreement on existence of a spectrum of dysplasia in the pre-invasive lesions among pathologists, accord on the number of tiers of grading has not been achieved. Some authors designate the term-CIS to the uppermost end of the spectrum where cells have all cytologic aspects of invasive carcinoma cells. Nevertheless, BilIN-1 and BilIN-2 are perceived to be of little clinical significance, whereas BilIN-3/CIS are typically associated with invasive carcinoma. As studies focusing on GB dysplasia are lacking, it is not currently known whether criteria exclusive to GB dysplastic lesions separate from those of bile duct are needed. More studies with application of BilIN classification in the GB are warranted for their validation.

One of the most challenging aspects of pathology reporting pertains to distinguishing high grade dysplasia/ CIS that extends into the Rokitansky-Aschoff sinuses (RAS) from an invasive carcinoma. Connection to surface and lack of desmoplastic response are pathological clues that indicate dysplasia affecting RAS rather than a T1b/T2 invasive carcinoma. Another tricky area is segregating low grade BilIN lesions from florid reactive atypia as causal factors for both lesions remain the same (such as gallstones, reflux etc.) and both lesions may cohabit. Epithelium with reactive changes caused by repair show a gradual transition between normal and abnormal cells in contrast to the abrupt transition seen in BilIN. Reactive changes may show pseudostratification, increased cellularity and slight nuclear enlargement. However, nuclei possess smooth contours and fine, homogenous chromatin. Intraepithelial neutrophils favor a reactive atypia. Immunohistochemically, dysplastic cells express cytoplasmic CEA, S100 A4 [108], CA19-9 [60] and diffuse nuclear p53 [109] and there is loss of p16 [110]; no single marker is useful as overlaps are frequent. Unlike the remaining GI tract, the GB is structurally unique in its lack of a well defined muscularis mucosae and a frequently fenestrated tunica muscularis. Further, extension of neoplastic proliferation into the RAS is commonplace. This, on many occasions, renders accurate classification of lesions into Tis, T1a or early T1b extremely challenging. To circumvent this dilemma, authors in high GBC risk regions, such as Chile and Southeast Asia, have introduced a collective terminology of ‘early gall bladder cancer’ for Tis, T1a or T1b neoplastic lesions.

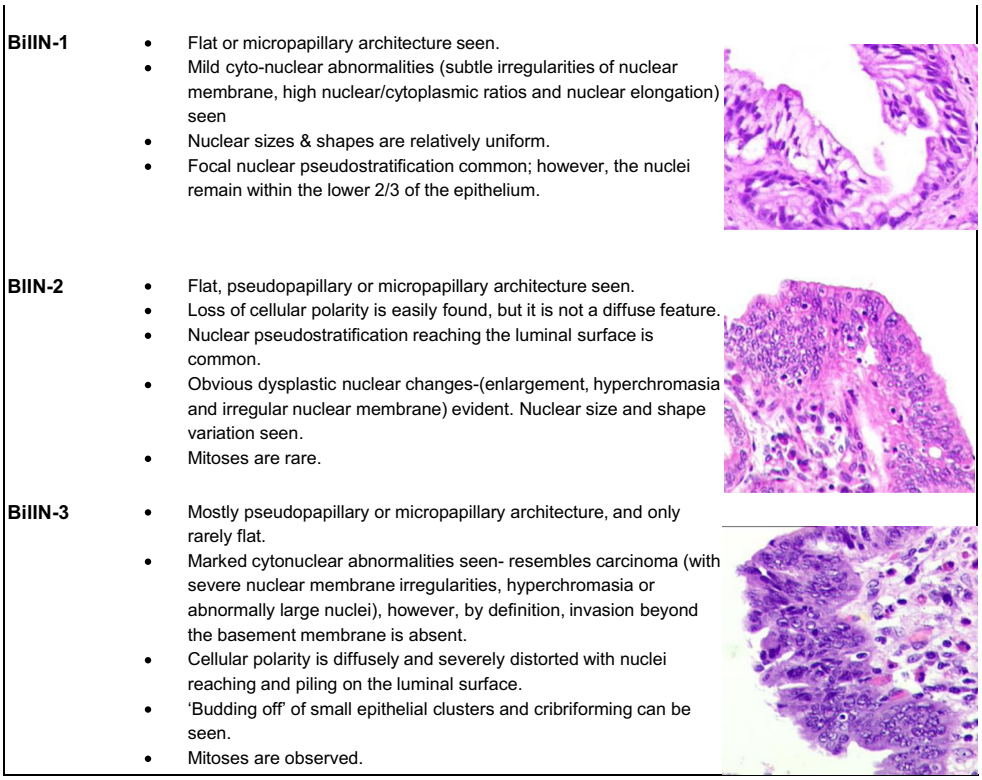

Fig. 1 Salient diagnostic and morphological features of biliary intraepithelial neoplasms (BilINs) of gallbladder [88]: BilIN-1, BilIN-2 and BilIN-3 (from top to bottom)

Cholecystectomy with negative margins is curative for BilIN-3/CIS. Finding BilIN, especially BilIN3, without invasive carcinoma in a cholecystectomy specimen warrants extensive and complete sampling. SEER (Surveillance Epidemiology End Results) database for CIS cases indicates a 100 % survival at 5 years while signals a drop to 70 % at 10 years. This conjectures a second malignancy surfacing due to a wide field cancerization phenomenon or an initially missed invasive focus due to inadequate sampling [111].

B) Tumoral precursor lesions

Recently, a unified terminology of intracholecystic papillary-tubular neoplasm of the GB (ICPN) has been proposed for all well defined, exophytic pre-invasive neoplasms that measure ≥1 cm [66]. All non-neoplastic polyps (benign fibroepithelial, fibromyoglandular, cholesterol, adenomyoma) irrespective of size, smaller metaplastic, dysplastic lesions or papillary in situ lesions<1 cm are excluded; flat papillary in situ lesions are considered to be a part of BilIN spectrum. ICPNs represent the GB counterparts of pancreatic IPMN or ITPN and bile ductal IPNB, and conceptually typify adenoma-carcinoma sequence. The criterion of ≥1 cm also concurs with the size criteria employed by surgeons to perform cholecystectomy for GB polyps. Large numbers of studies have made it sufficiently clear that sub-centimeter lesions are clinically inconsequential.

ICPNs are rare and their accurate incidence is difficult to assess due to limited data and definitional heterogeneity. However, incidence of<1 % is reported in cholecystectomy specimens whereas 5–23 % of invasive carcinomas reveal vestiges of a pre-existing ICPN [59, 62, 64, 78]. Adsay et al., in their systematic analysis of 606 invasive GBC, found 6.4 % invasive carcinomas originating in an ICPN [66]. Frequency of ICPN is higher in the women, with a mean age of occurrence being 60 years. Their association with gallstones has not been found to be strong. Occurrence in cases of Gardner's and Peutz Jeghers syndrome is recorded [112–114]. Often asymptomatic, their detection is usually incidental following a cholecystectomy. ICPNs exhibit diverse architectural patterns (papillary and tubular), grades of dysplasia (low to high), cellular differentiation; many times co-existing in the same lesion. Nearly half of the ICPNs have a papillary growth pattern [104]. On the basis of preponderant cell type, these are classifiable as [66]:

a. Biliary: commonest type (50 %); express MUC1.
b. Gastric, which has further two types:
    a) foveolar type (16 %)-uniform MUC5AC immunoreactivity is typical; it is commonly admixed with biliary type, and is accompanied by invasive carcinoma in approximately 60 % cases [66]
    b) pyloric type (20 %)- expresses MUC6:

- Mucinous type (reminiscent of pyloric gland adenomas).
- Non-mucinous type (Fig. 2d, e): This is emerging to be a distinct clinicopathologic entity. It has several distinctive features: forms large, multinodular fragile masses that detach and are readily dismissed as necrotic debris; clean background; tubular growth; non-mucinous cells with clear grooved nuclei, and β-catenin positive squamous morules; and lower frequency of invasive carcinoma (18 %) [66].

c) Intestinal: (8 %): immunoreactivity for CK20, CDX2, and MUC2 is seen.
d) Oncocytic (6 %) - MUC1 positive (contrasts with oncocytic IPMNs that are positive for HepPar1 and MUC6) [66].

MUC1 is seen in high grade dysplasia of any type; maybe a marker of high grade dysplasia.

Histological types of ICPN are depicted in Fig. 2. Except the pyloric non-mucinous ICPN, which is distinctive pathologically, other types are marked by hybrid morphology and heterogeneity in cellular lineage and immunophenotypic characteristics, further justifying a unifying term of ICPN.

Adsay et al. found invasive carcinoma in 55 % of ICPN cases [66]. Factors associated with invasion were extensive high grade dysplasia (i.e., >75 %), cell type (biliary) and papillary growth pattern [66]. Non invasive ICPN patients had 3- and 5-year survival as 90 and 78 % while invasive ICPN had 60 and 60 %, respectively [66]. In comparison to a median survival of 9 months in conventional pancreatobiliary GB adenocarcinoma, invasive ICPN had 35 months. This has been found independent of the stage of the tumors [66]. Prognostic significance of histological subtypes needs to be determined in larger studies. The adenoma-carcinoma sequence needs to be meticulously evaluated to elucidate the key genetic events and identify biomarkers for patient risk stratification.

Invasive Gallbladder Carcinoma

More than 90 % of GBC are adenocarcinoma [68]. Most GBC adenocarcinomas originate in the fundus (60 %), followed by body (30 %), and neck (10 %) [14]. Macroscopically, GBC can be infiltrative, papillary, nodular or infrequently, gelatinous. Alarmingly, in 10–37 % cases, tumor may not be apparent grossly [115]; alternately, when the tumor decides to spread extensively along the subserosa, the gross appearance may masquerade as a hyalinizing cholecystitis. This underscores an inherent grave hazard of missing a carcinomatous focus during sampling in unsuspected cases [115]. Pathologic examination of all GBs following routine cholecystectomy has been debated many times. At many places, surgeons routinely refrain from sending specimens for pathologic evaluation when gross examination is normal. Studies in support as well as against this practice abound [116–120]. Nevertheless, importance of a careful gross evaluation cannot be overemphasized.

Microscopically, adenocarcinomas are characterized by malignant glands marching unrestrained in a conspicuously dense desmoplastic stroma. Based on the degree of gland formation, adenocarcinomas are divided into: well differentiated (grade 1, >95 % gland formation), moderately differentiated

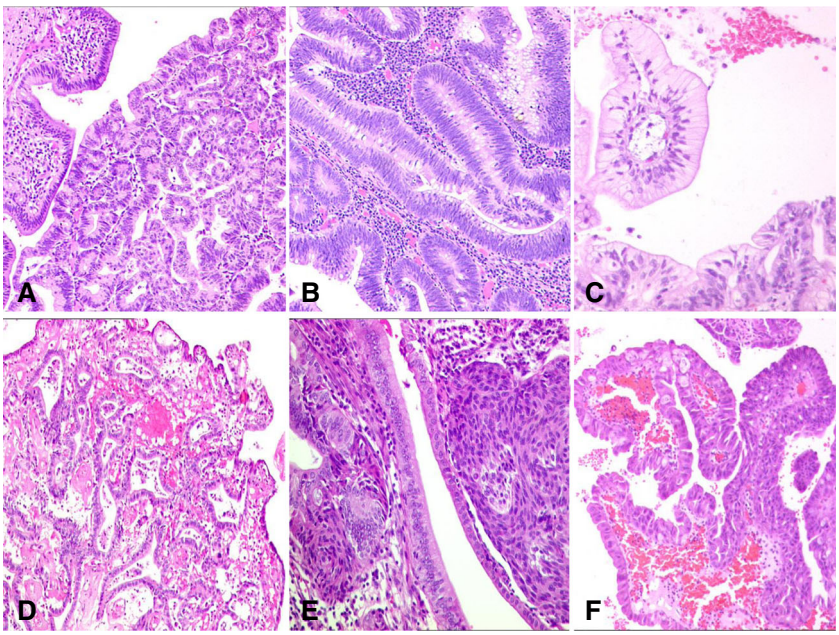

Fig. 2 Histology of intracholecystic papillary-tubular neoplasm (ICPN): **a**) Biliary type (H&E, X100x); **b**) Intestinal type (H&E, X200x); **c**) Gastric foveolar type (H&E, X200x); **d**) Gastric pyloric, non-mucinous type, low magnification (H&E,X100x); **e**) Higher magnification of gastric pyloric, non-mucinous, showing squamous morules (H&E, X200x); **f**) Oncocytic type (H&E,X100x)

(grade 2, 50–95 % gland formation) and poorly differentiated (grade 3, 5–49 % gland formation), and undifferentiated that lack gland formation (grade 4). Typical immunohistochemical (IHC) profile is: CK7, MUC1, CEA, CA19-9, and CK 20 (variable) expression. Scattered synaptophysin and chromogranin immunoreactive neuroendocrine cells are common within a conventional adenocarcinoma and should not invite a diagnosis of neuroendocrine tumor (NET).

Early GBC has an overall survival of approximately 90 %. Tumors with pathological Tis/pT1a/T1b with negative margins on cholecystectomy need no further treatment; pT2 tumors necessitate a radical cholecystectomy [121]. Adjuvant chemotherapy in patients with advanced pT3 or pT4 GBC is offered [121].

Variants

Uncommonly, histological variants of GB adenocarcinoma may be encountered by a pathologist. Awareness of their morphological features is imperative as the diverse histologic types carry varying prognostic import and therapeutic implications.

Papillary adenocarcinoma is typified by exophytic cauliflower like growth and is more inclined to pack the GB lumen rather than invade its wall. The papillae are formed of delicate fibrovascular cores lined by carcinomatous cells of biliary type, less commonly intestinal type (Fig. 3a). Invasive component is characteristically conventional tubular type. Historically, non-invasive papillary neoplasms have been lumped together with invasive papillary adenocarcinoma. Using the recently proposed classification, non-invasive lesions>1 cm are better classified as ICPNs with the uppermost end lesion labeled papillary CIS rather than papillary adenocarcinoma. This is endorsed by the fact that non-invasive tumors have a 10 year survival of 52 % compared to <10 % in patients with invasive tumors [122, 123]. Invasive papillary carcinomas are characterized by a less aggressive clinical course than conventional adenocarcinomas, mucinous and adenosquamous carcinomas [71, 111, 124].

Intestinal type is an unusual variant of adenocarcinoma identical in histologic appearance to colon adenocarcinomas, composed of glands with luminal necrosis, goblet cells and colonic type epithelium.

Clear cell adenocarcinoma (CCA) is identified by its eponymous clear cells that possess a glycogen rich clear cytoplasm, hyperchromatic nuclei, and well defined cell borders (Fig. 3b).

The cells may be arranged in nests, solid sheets and trabeculae [125]. This entity can mimic other clear cell neoplasms, which are however admittedly rare, and may invite differential diagnoses of metastatic clear cell renal cell carcinoma ma (CRCC), clear cell carcinoid and paraganglioma [126, 127]. IHC can resolve this; unlike CCA of the GB, CRCC is CK7-/CEA- and Vimentin+/CD10+. Clear cell carcinoid of the GB (an exceptionally rare neoplasm mostly arising in the setting of Von Hippel Lindau (VHL) syndrome wherein it is diffusely positive for inhibin) is positive for neuroendocrine markers whereas S100 protein decorates the sustentacular cells delineating the neuroendocrine tumor cells in paraganglioma [128].

Mucinous carcinoma (MC), defined as having more than 50 % extracellular (stromal) mucin, is very rare (2.5 % of GB carcinomas) (Fig. 3c) [129]. Signet-ring cells may be found floating within the mucin pools. Mucinous carcinomas appear

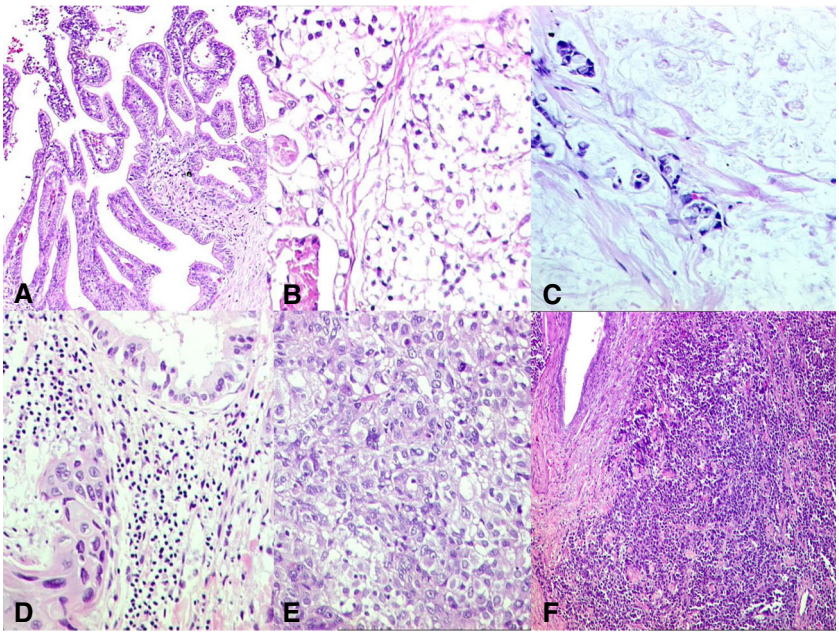

Fig. 3 Histologic variants of invasive gallbladder carcinoma. **a**) Papillary (H&E,X100x); **b**) Clear cell adenocarcinoma (H&E, X200x); **c**) Mucinous carcinoma (H&E,X200x); **d**) Adenosquamous carcinoma (H&E, X200x); **e**) Undifferentiated carcinoma with pleomorphic giant cells (H&E, X200x); **f**) Small cell carcinoma (H&E, X100x)

to have a set of unique clinicopathologic features: lack of female preponderance; clinical presentation with 'acute' symptoms; large and more advanced tumors at presentation (87 % are T3 vs. 43 % of conventional); and aggressive behavior [129]. IHC profile also appears to be distinctive: CK7 (57 %), MUC1 (57 %), MUC2 (86 %), MUC5AC (86 %), loss of E-cadherin (86 %), CK20 (29 %), CDX2 (14 %), and MUC6 (0 %). This subtype differs from conventional GB adenocarcinoma due to consistent MUC2 positivity, from pancreatic colloid carcinoma by CDX2 negativity and MUC1 positivity, from mammary colloid carcinoma due to lack of MUC6, from intestinal MCs by CDX2 and CK20 negativity and CK7 positivity and a more often microsatellite stable phenotype [129].

Signet-ring cell carcinoma, by definition grade 3, is predominantly composed of ring shaped cells possessing abundant intracytoplasmic mucin that displaces the nuclei to periphery. Rarely, signet-ring cells need to be distinguished from muciphages present in GB mucoceles. The neoplastic signet-ring cells are cytokeratin and CEA-positive whereas muciphages are negative for these markers. Metastases from the breast and stomach should also be ruled out when this subtype is detected in the biliary tract [68].

Adenosquamous carcinomas are uncommon, forming 4 % of all GBC [130]. They consist of malignant glandular and squamous elements with squamous component forming, by definition, 25–99 % of the tumor (Fig. 3d). A tumor with less than 25 % squamous component is considered as adenocarcinoma with focal squamous differentiation. Any glandular differentiation in a lesion that is predominantly squamous falls under the ambit of adenosquamous carcinoma. Pure **squamous cell carcinoma**s represent less than 1 % of all biliary malignancies. The squamous component is often associated with keratinization and expresses p63 and high molecular weight keratin while the glandular portion frequently produces mucin and usually shows CEA and B72.3 expression (Fig. 3d). Squamous component is high grade (presumably due to de-differentiation) and proliferates at a higher rate than the glandular component [130]. Squamous metaplasia has been detected in 12 % cases in the adjacent mucosa [130].

The biology of tumors with squamous differentiation is intriguing. The overall prognosis of adenosquamous carcinoma/squamous cell carcinoma is worse than that of ordinary adenocarcinomas. The tumors display an increased propensity for direct extension and early invasion into liver and neighboring organs [130–132].

Undifferentiated carcinomas are aggressive group of GBCs that histologically lack a definite direction of differentiation. It encompasses four types [60]:

Undifferentiated carcinoma, spindle and giant cell type (Fig. 3e) consists of variable proportions of spindle, giant and polygonal cells, but foci of well-differentiated neoplastic glands are usually uncovered after extensive sampling. Areas of squamoid differentiation may also be seen. The presence of cytokeratin in the spindle cells may help to distinguish this tumour from carcinosarcoma.

Undifferentiated carcinoma with osteoclast-like giant cells is an interesting entity that is histologically characterized by numerous multinucleated osteoclastic giant cells admixed with pleomorphic malignant cells. Analogous to pancreatic counterparts, the undifferentiated tumor cells are epithelial, thereby justifying the carcinoma label while the giant cells are reactive and non-neoplastic cells with phagocytic properties.

Undifferentiated carcinoma, small cell type is composed of sheets of round undifferentiated cells with vesicular nuclei and prominent nucleoli. This subtype needs distinction from lymphoma.

Undifferentiated carcinoma, nodular or lobular type comprises well demarcated nodules or lobules of neoplastic cells superficially resembling breast carcinoma.

Small cell carcinoma (Fig. 3f), high grade malignancy histologically identical to small cell carcinomas of the lung and GI tract, is quite rare with an incidence of 0.5 % of all GBC [124]. It has a tendency to metastasize early and is associated with a dismal outcome. Admixture with adenocarcinoma or squamous cell carcinoma is not uncommon, however, generally does not alter outcome. The most common site of metastases is lymph nodes (70 %), followed by liver (64 %), and lungs (10 %). The 5- and 10-year relative survival rates of small cell carcinoma of the gallbladder are 8 and 0 %, respectively [127].

Molecular Pathology

A lot of research has been directed towards unearthing molecular genetic events that are critical for GB carcinogenesis and progression; however, existing knowledge is still primordial. Elucidation of underlying genetic events offers the promise of a breakthrough in discovery of biomarkers and novel therapeutic targets. From a multitude of genes studied in GB carcinogenesis, a few key players are discussed briefly and summarized in Table 2.

Oncogenes

KRAS proto-oncogene encodes a protein GTPase that is an initial player of innumerable crucial signal transduction pathways. KRAS activating point mutations have been reported in the second nucleotide of codon 12, majority of which are attributable to a G to A transition at this nucleotide, resulting in the substitution of aspartic acid for glycine [133, 134] that engenders de-regulated and inappropriate signaling. A wide range of frequency of KRAS mutations with geographical

Table 2 Summary of genetic alterations in gallbladder cancer

Oncogenes		Frequency	Features	References
	KRAS	10–67 %	Mutation at invasive stage; high incidence in ABPDJ patients	[135–141]
	HER2	Variable; 33–64 %	Amplification in upto 70 %	[142–144]
	BRAF	33 %	Little data available	[189]
	EGFR	15 %	Over expressed in dysplasia and GBC	[146]
	Cyclin D1	41 %	Associated with lymphovascular invasion	[149]
	Cyclin E	49 %		[150]
Tumor suppressor genes				
	TP53	31–100 %; most studies >50 %	Earliest change; detected in 1/3rd of normal and dysplastic epithelia	[109, 141, 152, 153, 155, 156, 158, 159]
	p16/$^{CDKN2A/INK4A}$	10–50 %	Late change at CIS stage; inactivation is associated with adverse prognosis	[165, 166]
	p21/^{CDKN1A}	49 %	P21 expression with other genes, p53 and p27, correlates with survival	[167]
	FHIT	75 %	Late change at CIS stage	[169]
	Mitochondrial DNA	–	Early alteration	[171]
Angiogenic /inflammatory pathway genes				
	COX-2	59–80 %	Early involvement; Expression associated with worse prognosis	[172, 173]
	iNOS	71 %	Induces early alterations	[174]
	VEGF	55 %	Increased angiogenesis	[173]
Adhesion molecules				
	CD44v3 and CD44v6	Approximately 50 %	Late expression	[177]
	Beta-catenin	–	Associated with better prognosis	[179, 180]
	CD54	39 %	Late stage	[181]
	Claudins	–	Reduced expression in GBC	[176]
	EpCAM	–	Over expressed in GBC	[177]
Mucins				
	MUC-1	81 %	Associated with poor survival	[182]
	MUC-4	55 %	Associated with poor survival	[185]
DNA repair				
	MGMT	60 %	Associated with liver invasion and poor prognosis	[186]
	MSI-high phenotype	83 % in dysplasia	Early change	[59]
Telomerases				
	hTERT	93 %	Early event	[187]

heterogeneity is reported in GBC, ranging from 10 to 67 % [135–141]. A conspicuously high rate of KRAS mutation is detected in early lesions in APBJ malformation patients in Japan (50–83 %) and is also promulgated as a diagnostic marker for GBC in this condition [135, 136]. Correlation with stage, histology or survival has, however, not been found [136].

HER-2/neu (ERBB-2) proto-oncogene encodes transmembrane receptor tyrosine kinases (RTK) that play a key role in co-regulation of DNA repair, apoptosis and cell cycle check points. HER-2 oncogene overexpression ranges from 33 to 64 % cases of GBC; while amplification is identified in upto 70 % GBC cases [142–144]. Overexpression of HER-2/*neu* is important for carcinogenesis of gallbladder cancers, and detection of the above abnormalities in bile is helpful for early diagnosis [145].

Epidermal growth factor receptor (EGFR or HER1) is a member of erbB family of proteins that encode RTKs and is involved in signal transduction leading to DNA synthesis and cell proliferation. Somatic mutations of the (EGFR) tyrosine kinase domain are found in approximately 15 % of the biliary tract and GBC [146]. A greater over-expression of EGFR was found in GBC (70.7 %) and dysplasia (85.7 %) than in simple hyperplasia (27 %) and normal gallbladder (0 %) [147, 148]. Genetic alterations in HER2 and EGFR indicate that specific monoclonal antibodies or tyrosine kinase inhibitors may have a therapeutic role in the treatment of GBC in future.

Cyclins, cyclin D1 and cyclin E, promote cell cycle progression. Overexpression of cyclin D1 and cyclin E is seen in 41 and 49 % cases of GBC, respectively [149, 150]. Cyclin D1 overexpression has been found to be associated with lymphatic and venous invasion [151].

Tumor Suppressor Genes

TP53 gene is known to be consistently inactivated in GBC [152–156]. Most frequent mutations in GBC are missense mutations that lead to accumulation of a non-functional protein with lengthened half-life that is detectable on IHC [157]. The reported frequency of TP53 mutations is wide, ranging from 31 to 70 % in some [152, 153, 155, 156] and 59–100 % in others [141, 158, 159]. Geographical fluctuation in incidence is not apparent. A study from high-incidence areas, Chile and Japan reported 59 and 60 %, respectively [156]. Mutation involving G to A transition is seen most frequently worldwide. This is similar to mutations found in other inflammation-driven carcinomas i.e., cancers arising in UC and Barrett's esophagus [160, 161]. Additional 2 mutations unique to Japanese cases are G to C and G to T transversions [154, 162]. Allelic loss is an early event, detected in the normal epithelium [135]. Variation with histology is also reported; 100 % in intestinal, 66 % in papillary type, 83 % in adenosquamous, and 66 % in giant cell carcinoma [163]. No correlation has been found between p53 immunoreactivity (found in 35–92 % GBC cases) and prognosis or recurrence.

p16/$^{CDKN2/INK4A}$ abrogation by inactivation (41 %), loss of heterozygosity (11 %) and methylation (24 %) is reported in GBC [164]. Diminishing immunoreactivity with increasing malignant transformation is observed: normal epithelium (50–90 %), dysplasia (50 %) and adenocarcinoma (10–50 %). p16 inactivation is associated with an adverse prognosis [165, 166]. Incidence of p16 loss is inversely proportional to RB inactivation

p21/^{CDKN1A} is a cyclin dependant kinase inhibitor important in cell cycle regulation. Reduced expression is seen in 49 % GBC [167]. In patients with p53 mutation, loss of p21 is associated with longer survival [168]. In patients with intact p27, loss of p21 correlates with better survival rate [168].

Fragile Histidine Triad (FHIT) is a tumor suppressor gene located at 3p14.2 and encodes an enzyme that hydrolyses diadenosine nucleotides into ADP and ATP. Methylation of promoter, LOH, frameshift mutations are universal in GBC cases; loss of expression seen in 75 % GBC [169]. Changes are detected early in the sequential development of GBC [170]. **Mitochondrial DNA (mt DNA 310)** somatic mutations at the displacement loop, presumably induced by reactive oxygen species, are identifiable at early stages of GBC [171].

Cyclooxygenase-2 (COX-2) is induced by cytokines, mitogens and growth factors, and it elaborates prostaglandins, promotes cell growth and induces neovascularisation. Expression of COX-2 in normal (14.3 %), dysplastic (70.3 %), and adenocarcinoma (59–80 %) indicates early involvement in GB carcinogenesis [172, 173]. **Inducible nitric oxide synthetase (iNOS)** expression is seen in 88 % of chronic cholecystitis and 71 % of adenocarcinoma [174]. Vascular endothelial growth factor (VEGF) is expressed in 54.7 % in GBC and overexpression correlates with neovascularisation [173].

Adhesion molecules are cell surface proteins involved in binding with neighbouring cells as well as the extracellular matrix. Cell junction regulatory proteins, claudins, are

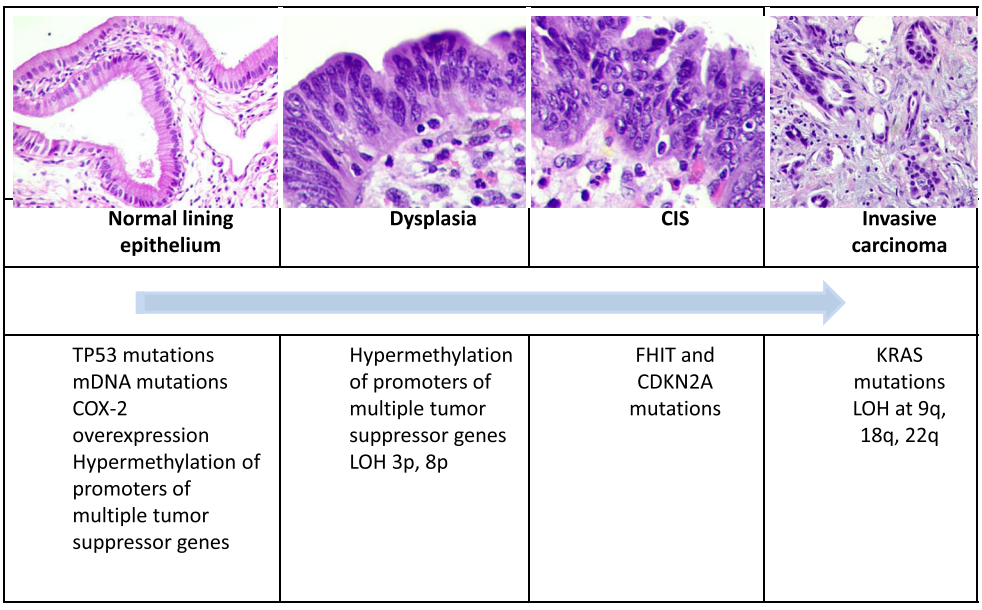

Fig. 4 Sequential morphological and genetic alterations at different stages of gallbladder carcinogenesis

implicated in the process of carcinogenesis and tumor progression [175]. Claudins', especially claudin 10, reduced immunohistochemical expression is seen in GBC [176]. EpCAM is ubiquitously expressed in all GBCs and is an independent prognostic marker. Abnormal CD44 expression is seen in nearly 50 % of subserosal GBCs [177]. High expression of CD44 variants, CD44v3 and CD44v6 is seen in GBC and is absent in normal mucosa [178]. Expression is seen in late stages. Cytoplasmic and nuclear expression of beta-catenin is associated with better prognosis [179, 180]. Intercellular adhesion molecule-1 (CD 54) is observed in adenoma (14 %) and adenocarcinoma (39 %) [181].

Mucins are high molecular weight glycoproteins on cell surface, involved in cellular cross-talk, signaling and metastasis. Normal GB mucosa lacks MUC-1 expression whereas a de-polarised expression seen in invasive adenocarcinoma [182]. MUC-1 overexpression is correlated to lymphatic invasion, tumor progression and poor survival in GBC [183, 184]. MUC-4 expression, seen in 55 % of GBC, is found to be associated with poor survival [185].

Microsatellite instability (MSI) is variably reported in GBC, approximately in 10 % Chilean patients [59]. MSI-high phenotype associated with initial stages of GBC; seen in 33 % intestinal metaplasia and 83 % dysplasia. **O6-methylguanineDNAmethyltransferase (MGMT)** is detected in 60 % GBC and correlates with increased liver invasion and poor prognosis [186].

Telomerases (catalytic subunit of telomerase (hTERT) are progressively expressed in normal epithelium (3 %), regenerative epithelium (4 %), low grade dysplasia (25 %), high grade dysplasia (82 %) and adenocarcinoma (93 %) [187]. hTERT re-expression reflects an early event in the multistep progression of GB carcinogenesis.

Although countless molecular events and myriad genetic alterations are increasingly being reported in various stages of GBC, many pieces of this puzzle are still missing. However, what is emerging is that GB carcinogenesis is multi-step process orchestrated by sequential alterations (genetic and epigenetic) involving a multitude of genes from diverse signaling pathways. Orderly acquisition of genetic alterations is to a large extent in tandem with recognizable morphological changes (Fig. 4). The early genetic alterations appear in the histologically normal looking epithelium of inflammation associated mucosa. These early changes include TP53 mutations, COX-2 overexpression, mitochondrial DNA mutations and hypermethyaltion of promoters of various tumor suppressor genes. During further development of dysplasia, changes include allelic losses of several chromosomal loci (especially 3p and 8p). Late changes at CIS stage include inactivation of FHIT and CDKN2A and losses of additional chromosomal regions, especially at 9q, 18q and 22q. Finally, KRAS mutations presage invasive tumors [188]. Based on allelic losses and patterns of genomic instability, it has been established that many molecularly transformed clones are present in histologically normal and dysplastic mucosa adjacent to GBC indicating a field change [189].

Conclusions

Gallbladder carcinoma remains a notoriously fatal cancer with abysmal prognosis. However, gaps in our comprehension of the complex mechanisms operative in GB carcinogenesis are being increasingly filled. An intimate interplay of genetic, environmental and life-style factors operative in GBC etiopathogenesis is now acknowledged. Pre-cancerous lesions of the GB have received considerable attention recently that has led to their better characterization. Proposal of a unified terminology promises to be giant step in bringing the previously disjointed pathology taxonomy of pre-invasive lesions, together. The underlying sequence of molecular events occurring parallel to these distinctive pre-invasive histological stages, are being uncovered. This underscores an urgent need to improve methods of clinical detection of these pre-cancerous lesions in order to allow effective prevention of GBC. Augmented awareness of GBCs deceptive gross appearances have led to an increasing realization of a protocol based sampling of specimens to avoid under- or missed sampling of early lesions or even cancer. Recently gained insights into the genetic alterations crucial in GB carcinogenesis offer tremendous optimism in exploring role of newer biomarkers (for risk stratification and prognostication) in future. Since GBC is an inflammation-driven cancer, anti-inflammatory therapeutic approaches, which can block/modify carcinogenic mechanism/s, such as use of antibiotics, anti-inflammatory agents or cellular microRNAs, also need to be investigated. Affecting economically deprived parts of the world, GBC often fails to attract the medical and research funding and attention it deserves. To overcome these challenges, a collaborative effort between the developed and developing worlds, perhaps, is also a need of the hour.

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