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PATHOBIOLOGY OF GALLBLADDER CANCER

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Abstract

Carcinoma of the gallbladder (GBC) is the most common malignant tumour of the biliary tract. Gallbladder cancer demonstrates considerable geographic and gender variation in incidence varying greatly in different parts of the world. While it is more frequent in northern and eastern India, Japan, Chile, China, Eastern Europe and South America, its prevalence is relatively low in many western countries. It affects women two to six times more frequently than men. The incidence of GBC increases steadily with increase in age. Carcinoma of gall bladder is the fifth most mortality causing cancer and its cases are alarmingly increasing in south-east Asia. It is one of the obesity associated cancers and positively correlates with prolonged cholelithiasis (gallstone) and cholecystitis. Other risk factors include gallbladder polyps, anomalous pancreaticobiliary duct junction, chemical carcinogens, and chronic infections. Despite being first described more than two centuries ago in 1777, there has been little progress in its early diagnostics, prognosis and effective treatment. Non-random genomic alterations, gene mutations and epigenetic modifications have been reported in a few studies. Here we have reviewed our current understanding of the epidemiology, aetiology and genetic and epigenetic alterations in GBC.

Epidemiology

The highest figures are reported from the populations of, northern and eastern India, Japan, Chile, China, eastern Europe, native America and South America (Schottenfeld and Beebe-Dimmer, 2006; Mishra *et al*, 2003). While it is uncommon in Europe and the United States, it is more frequent in Chile, Bolivia and Israel (Orth and Berger, 2000). The incidence of carcinoma gall bladder in India ranges from 1.01 per 100000 for males to 10.1 per 100000 for females (Indian Council of Medical Research (ICMR) Annual Report of population-based cancer registries of the National cancer Registry Programme, 1996) but the actual number may be much more in the endemic zones of Western Bihar and Eastern Uttar Pradesh where it is the third commonest malignancy of the alimentary tract (Shukla *et al*, 1985). The age adjusted incidence rate for carcinoma of the gallbladder is low in most of the Indian Cancer Registries, with a mean of 2.3/100,000

for females and 1.01/100,000 for males. The incidence is 10-12 times higher in Northern India (5.7/100,000) women) as compared to Southern Indian (0-0.7/100,000 women). Delhi and Bhopal have the highest incidence rates in India being 6.6 and 5.2 for females and 1.9 and 2.2 for males per 100,000 respectively. Incidence of gallbladder cancer is very high in northern India, 4.5 per 100,000 for males and 10.1 per 100,000 for females, but low in South India, 1.2 per 100,000 for males and 0.9 per 100,000 for females as reported by the population based cancer registry. Shukla et al, (1985) reported a male to female ratio of 1:2.5 in their series of 315 patients including 225 females, and the disease was more common in 4th and 6th decade of life with an average of 50 years in and around Varanasi. In the United States of America, Singapore and Nigeria, it is an uncommon cancer with an incidence of 2.5 per 100,000. In contrast, Chile has a much higher incidence of 7.5 per 100,000, especially in American Indian females (Wanebo and Vezeridis, 1993). Chile has the highest incidence of gall bladder cancer and this incidence has been regularly increasing over the years.

The lowest incidence is found among the black Rhodesians, black Americans and Spaniards. Its incidence varies from 27/ 100,000 in Chile to 1/100,000 in US. Incidence of carcinoma of the gallbladder on the basis of the population based cancer registries was 11.6/100,000 for males and 13.41100,000 for females in Japan (Onoyama *et al*, 2002). The gallbladder cancer increases considerably with age. The mean age of patients with carcinoma of the gallbladder is 65 years and the highest incidence of the disease occurs in the seventh and eighth decades of life (Piehler and Crichlow, 1978). Carcinoma of the gallbladder affects women 2-6 times more frequently than men, although the extent of this bias varies in different geographical regions (Lazcano-Ponce et al, 2001). Women are 2-6 times more likely to develop gallbladder cancer than men in the United States. The mean age of the patients with gallbladder cancer is 65 years (55 - 82 years). The large geographic differences in the incidence of carcinoma of the gallbladder suggests the presence of nutritional and environmental factors in the etiology of the malignancy, in addition to genetic, ethnic, racial and social factors (Diehl, 1983).

Etiology

The presence of gallstone (cholelithiasis) is one of the major risk factor of GBC. The cause and precise mechanism of origin and formation of gallstones are unknown. Association of gallstones with gallbladder carcinoma has been well established (Zantonski *et al*., 1997). It is not clear whether the association represents a causal link or the presence of common risk factor. Evidence in favour of a link between these diseases is substantial: gallstones are found in 65–90% of patients with gallbladder carcinoma (Misra et al., 1995) and the risk of developing gallbladder carcinoma proportionally to gallstone size. The latter exemplified by the relative risks of gallbladder carcinoma for patients with gallstones, relative risk is 2·4 if the stones are 2·0–2·9 cm in diameter, but increases to 10·1 if the stones are larger than 3·0 cm (Towfigh *et al*., 2001). However, several other factors may be important in the development of gallbladder carcinoma because about 10–25% of patients with this disease do not have associated cholelithiasis and only a small proportion (1–3%) of patients that do have gallstones actually develop cancer.

Porcelain gallbladder, the pathological finding of a brittle gallbladder with bluish discolouration resulting from extensive calcification of the organ wall, has also been associated with carcinoma in 12·5–62% of patients (Towfigh *et al*., 2001). Less extensive calcification can be detected by ultrasound examination. Recent reports suggest that there is a much lower incidence of gallbladder cancer associated with diffuse intramural calcification (type I; complete) than with selective mucosal calcification (type II and III; incomplete) (Stephen *et al*., 2001).

The presence of polyps is another predisposing factor for carcinoma of the gallbladder. Recent evidence suggests that polyps larger than 10 mm in diameter have the greatest malignant potential. If diagnosed in asymptomatic patients, even in the absence of gallstones, removal of the gallbladder is recommended (Aldridge *et al*., 1990). Small polyps (less than 10 mm in diameter) need only be removed if they are producing symptoms or are associated with gallstones.

Anomalous Pancreaticobiliary Duct Junction (APBDJ) is associated with the development of gallbladder carcinoma (Henson *et al*., 1992). This anomalous ductal union is seen in about 17% of patients with carcinoma compared to less than 3% of patients with other hepatobiliary disorders. The APBDJ between the common bile duct and pancreatic duct is not under the control of the sphincter when outside the duodenal wall, therefore, pancreatic juice can freely flow back into the gallbladder causing bile stasis, leading to precancerous changes in the gallbladder mucosa. Patients who have APBDJ without dilatation of the bile duct develop gallbladder stasis. These patients have a high incidence of gallbladder carcinoma than patients with bile-duct dilatation (Chijiiwa *et al*., 1995). Patients who develop carcinoma of the gallbladder in association with APBDJ are generally young and have a low incidence of gallstones.

Methylcholanthrene, O-aminoazotoluene, and nitrosamines cause gallbladder carcinoma in experimental animals (Albores-Saavedra *et al*., 1986). Occupational exposure to chemical carcinogens in individuals working in the rubber industry suggests a possible role for these compounds in gallbladder carcinogenesis. In north India, the use of mustard oil loaded with carcinogenic impurities has been suggested as an etiological factor (Hai *et al*., 1994). High concentration of free radical oxidation products and secondary bile acids has been reported in patients with gallbladder carcinoma compared to a control group of patients with cholelithiasis (Shukla *et al*., 1993;1994; Pandey *et al*., 2000).

Persistence of infection leading to chronic inflammation, and production of certain toxins and metabolites with carcinogenic potentials, by certain bacteria has been speculated to be involved in the transformation of the gallbladder epithelium. Therefore, any bacteria that have evolved to acquire both of the above carcinogenic mechanisms can cause cancer. *Salmonella typhi* has been found to be prominently associated with CaGB. Chronic typhoid carriage (persistence) and production of mediators of chronic inflammation and a genotoxic toxin (cytotoxic distending toxin, CdtB) are also known for this bacterium. Furthermore, the natural concentrating function of the gallbladder

might amplify the carcinogenic effect of the mediators of carcinogenesis. In addition to *S. typhi*, certain species of *Helicobacter (H. Bilis and H. hepaticus*) and *Escherichia coli* have also been implicated in carcinogenesis. As the isolation rate is very poor with the presently available culture techniques, the existence of bacteria in a viable but non-cultivable state is quite likely; therefore, sensitive and specific molecular techniques might reveal the etiological role of bacterial infection in gallbladder carcinogenesis. If bacteria are found to be causing cancers, then eradication of such infections might help in reducing the incidence of some cancers (Nath *et al*, 2008; 2010).

Sign, symptoms and histologic types of gallbladder carcinoma

GBC is in general a late presenting disease. It does not show defined and specific sign and symptoms. Hence, there are no well established criteria for precise and early diagnosis. These nonspecific symptoms have been grouped into five clinical syndromes (Piehler *et al*.,1978). The first of these is acute cholecystitis−about 1% of patients for acute cholecystitis have an earlier stage of carcinoma and they have improved survival. Second is chronic cholecystitis. The third syndrome is biliary-tract disease with symptoms of jaundice, weight loss, general weakness, and pain in the right upper quadrant. Patients with this clinical syndrome have extensive disease. The fourth category refers to malignant tumours outside the biliary tract, with symptoms of anorexia, weight loss, general weakness, and local complications of the tumour such as a fistula or invasion of adjacent organs. The last category is benign manifestations outside the biliary tract, the small group of patients with this syndrome present with gastrointestinal bleeding and upper gastrointestinal obstruction.

Gallbladder carcinoma usually produces asymmetric thickening of the gallbladder wall with infiltration to surrounding structures. Most cancers originate in the gallbladder fundus. As the tumour progresses, the gallbladder may fill with tumour or may contain pus, mucus, or stones. Early carcinomas can appear as a mucosal plaque, a polypoid or papillary outgrowth, or discrete thickening of the wall (Campbell et al., 1998). There are papillary, tubular, and nodular forms of GBC (Joffe *et al*., 1981). Papillary tumours are less likely to invade the liver directly and have lower incidence of lymph-node metastasis. Most carcinomas of the gallbladder are adenocarcinomas (80–95%), and can be papillary, tubular, mucinous, or signet cell type. Less common types of GBC are undifferentiated or anaplastic carcinoma (2–7%), squamous cell carcinoma (1–6%), and adenosquamous carcinoma (1–4%) [17]. Clear cell adenocarcinoma of the gallbladder is composed of cords, sheets, nests, and trabeculae of clear cells with well-defined cytoplasmic borders (Vardaman *et al*., 1995) and may be confused histologically with metastatic renal cell carcinoma. However, clear cell adenocarcinomas may also contain areas mixed with conventional adenocarcinoma and mucin production, findings that help distinguish these tumors from renal cell carcinoma.

The remaining epithelial cell types occurring in the gallbladder include adenosquamous carcinoma, squamous cell carcinoma, small (oat) cell carcinoma, and undifferentiated carcinoma. The adenosquamous carcinoma contains a mixture of

malignant glandular and squamous components. The pure squamous cell carcinoma constitutes only 1% of all malignant gallbladder tumors and consists of cords, islands, or sheets of malignant squamous cells separated by dense fibrous stroma (Albores-Saavedra *et al*., 1986). These tumors most likely arise in areas of previous squamous metaplasia, and their histologic features may vary from anaplastic to well-differentiated, keratinizing squamous cell carcinoma. Small (oat) cell carcinomas of the gallbladder are rare and highly aggressive tumors.

They are histologically identical to small cell carcinomas of the lung and gastrointestinal tract. Paraneoplastic syndromes may be associated with small cell carcinoma of the gallbladder, and Cushing syndrome has been reported in association with a corticotropin-secreting apudoma of the gallbladder (Spence *et al*., 1975).

Genetic and molecular genetic alterations in gallbladder carcinoma

The genetic studies in cancer have provided important clues in our understanding of the molecular mechanisms of tumour development. Genetic mutations accumulate in a sequential manner during tumour progression. Cytogenetic and molecular genetic studies including high resolution mapping using sequence tagged repeat polymorphic markers have revealed multiple genetic alterations in GBC. Non-random losses at 3p, 4q, 5q, 9p, 10p, 10q, 11p, 14p, 14q, 15p, 17p and 21p were reported by Goruneova *et al.* (1999). Loss of heterozygosity (LOH) using sequence tagged repeat polymorphic markers have been demonstrated on chromosomal arms 1p, 3p, 5p, 5q, 6q, 8p, 9p, 9q, 13q, 16q, and 17p in gallbladder carcinoma (Chang *et al.* 1999; Kim *et al.* 2001; Arakawa *et al*. 2004; Kuroki *et al*. 2005) while LOH on 13q and 18q is frequent in higher grade (stage III and IV) gallbladder carcinomas (Chang *et al.* 1999). LOH in dysplasias on chromosomal arms 3p, 5q, 9p, 13q, 13q, 16q and 17p suggest that these are early changes in the pathogenesis of GBC (Chang *et al.* 1999; Kuroki *et al.* 2005). Several of the LOH loci correspond to the sites of tumour suppressor genes viz., 1p34-36 (p73, p53-related gene), 3p25 (VHL), 3p24 (RARβ), 3p21.3 (RASSF1A), 3p14.2 (FHIT), 5q21 (APC), 8p21-23(PRLTS, FEZ), 9p21 (p15 and p16), 9q (DBCCR1), 13q14 (RB), 16q24 (WWOX, FRA16D) and 17p13 (p53) (reviewed in Kuroki et al, 2005). In a limited panel of tumours Yoshida *et al*. (2000) have demonstrated LOH at p53, DCC, APC, RB and NM23-H1 tumour suppressors. LOH at 17p increase with the stage of tumour and correlates with the metastasis, it is more frequent in GBC with metastasis than without metastasis (Chang *et al*. 1999).

In a study of a panel of 169 microsatellite markers spanning all non acrocentric autosomes and X demonstrated LOH at 21 chromosomal arms including 7q, 11q, 12q, 18q, 19p, 22q and Xq in addition to the above mentioned chromosomal arms (Wistuba et al, 2001).In a relatively recent study, homozygous deletion of the exon 2 of p16 with frequent LOH at 9p21-22, promoter hypermethylation of p16 and corresponding down regulation of p16 was demonstrated while no p16 mutation was detected suggesting deletion and promoter methylation as major mechanism of inactivation of the tumour suppressor p16 (Caca *et al*. 2002; Tadokora *et al.* 2007).

Although microsatellite instability (MSI) is a rare event in GBC, it has been demonstrated to have a role in gallbladder carcinogenesis. While MSI has a limited role in GBC, it has inverse correlation with LOH suggesting that LOH and MSI are two independent pathways in its pathogenesis (Chang *et al*. 1999; Yoshida *et al*. 2000; Kim *et al*. 2001).

Role of activating/inactivating mutations of oncogenes/tumour suppressor genes have been demonstrated in the oncogenic transformation of GBC, however, few of these are shown in dysplasias or adenomas. K-ras, p53 and p16 mutations were shown in gallbladder carcinoma but not in dysplasias or adenomas (Jonas et al, 1997; Yokoyama *et al*. 1998; Kim *et al*. 2001; Seatta *et al*. 2001). Rashid *et al*. (2001) demonstrated high expression of K-ras and p53 with high MSI and alteration of TGFβRII gene. Also, overexpression of p53 with low expression of p16 and RB has been shown (Parwani *et al* 2003) by immunohistochemistry. RAS/RAF/MEK/ERK kinase cascade is activated in GBC by either K-ras mutation or B-raf mutation along with p53 mutations (Saetta *et al*. 2004) leading to aberrant Nrf2 transcription factor activity. However, K-ras and B-raf mutations were not observed together but p53 mutation could be observed with either of the two gene mutations. B-raf mutations were mostly at the hot spot codon 599 of exon exon 15. Additionally, Kaep1 mutation that constitutively activates Nrf2 and has prognostic value by conferring resistance to chemotherapy (Shibata *et al*. 2008). Mutations in k-RAS have been reported in 39–59% of patients with gallbladder carcinoma although a higher frequency (50–83%) has been reported in patients with associated APBDJ. The relation with gallbladder stones suggests differential pathways of gallbladder carcinogenesis in patients with and without stones (Misra *et al*., 2000).

Epigenetic alterations are the hallmark of various kinds of tumours including GBC. Promoters of several genes have been demonstrated to be hypermethylated. Inactivation of RASSF1A by promoter hypermethylation is an important mechanism of disruption of K-ras dependent cell cycle regulation for K-ras wild type tumours (Kuroki *et al*. 2005). In a significant study that reveals methylator phenotype demonstrated promoter hypermethyaltion of p16INK4a, MGMT, hMLH1, E-cdherin, DAPK associated with mutations in p53, K-ras, β-catenin, and p16INK4a (Koga *et al.* 2004). Most of the mutations in this study were GC to AT transition (58%) and associated with MGMT methylation and thereby its inactivation. MGMT catalyses the removal of unusual methyl group from methyl-guanine that base pairs with adenine during replication introducing GC to AT transition. Besides, under-expression of MGMT and mismatch repair genes hMSH1 and hMSH2 was demonstrated by Kohya *et al*. (2002) related to poor prognosis suggesting the role of unrepaired/underrepaired DNA damages in the carcinogenesis of gallbladder. Hypermethylation of p16 and p14 promoters at 9p21 (frequent site of LOH) suggests that methylation plays critical role as second hit in the carcinogenesis of gallbladder (Caca *et al.* 2002; Klump *et al*. 2003). Role of genetic polymorphisms have also been associated with GBC. Two polymorphisms at the promoter of Cholecytokinin A receptor (CCK-AR) affects its methylation and is associated with its reduced expression levels (Miyasaka *et al*. 2002). Takahashi *et al*. (2004) demonstrated aberrant promoter methylation of multiple genes in GBC that showed correlation with clinical

status. While HPP1 was more frequently methylated in male pateints (55%) than in female patients (10%; P = 0.004), tumours displaying exclusively papilliary or mixed papilliary/tubular patterns demonstrated higher frequency of APC methylation than in cancers exhibiting only tubular features 60% versus 13% respectively, P = 0.004). Interestingly, methylation of RIZ1 correlated with tumor depth of invasion in the gallbladder wall and in adjacent liver tissue (P = 0.0037 and P = 0.024, respectively); and CDH1 methylation was associated with the presence of distant metastasis (P = 0.024).

Conclusion

Carcinoma of the gallbladder has a limited epidemiology with diverse geographical locations suggesting a larger role of environment including lifestyle tightly linked to genetic factors. Strong association of gallstone indicates that cholelithiasis and associated inflammatory responses might play a role in the initiation of the disease. Since gallbladder is the concentrating organ, accumulation of toxic waste may act as carcinogens and play a role in oncogenic transformation. Genetic information has revealed a plethora of alterations from gross losses to individual gene mutations and epigenetic modifications. Differential mutational status suggests that more than one parallel/distinct pathway are involved in the initiation and progression of the disease. However, there is limited information about its sequential progression. Currently there are few diagnostic and prognostic markers for GBC.

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