RESEARCH

A genetic roadmap of gallbladder cancer disparities: a potential for development of targeted therapies

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ABSTRACT

Gallbladder cancer (GBC) is an aggressive disease with a dismal prognosis and resistance to chemotherapy. Due to difficulties in early diagnosis of GBC, about 90% of patients are detected at advanced stages with palliative care being the only viable option. While surgery remains the mainstay treatment for early GBC, most patients who undergo surgical resection suffer from the high rates of recurrence. Unfortunately for many patients, surgical resection is not considered a possibility due to the stage at which they are diagnosed. Adjuvant therapies in the form of radiation and/or embolization of the tumor have been probed for the disease with moderate success. While there have been multiple studies (both retrospective and pooled analysis) that suggest an added advantage of combining adjuvant therapy with surgical resection, data from prospective studies is limited. Interestingly, GBC affects women, American Indians, Alaska Natives, black people, and certain ethnic groups in peculiar geographic locations such as Chile, India, China more than other groups elsewhere. These disparities in gender and ethnicities demonstrate the need for better understanding of underlying genetic events of GBC so that molecularly targeted therapies could be developed to provide hope for improving treatment response and better outcome. However, for GBC not much progress has been made mainly due to the lack of understanding of molecular pathogenesis of this disease. This article presents a review of literature focused on molecular and genetic alterations in GBC, and as to how effective targeted therapeutic strategies can be developed with demonstrated survival benefit.

KEYWORDS: gallbladder cancer, GBC, gallstone, genetic polymorphism, mutation, targeted therapy, molecular pathogenesis, health disparity

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Introduction

Early detection of GBC has been challenging despite advancements in medical imaging technologies such as Contrast Tomography (CT) and Ultrasound scanning. The majority of the cases are detected incidentally during surgery for cholelithiasis and hence detected at very advanced stage of the disease. GBCs are the commonest biliary tract cancers worldwide. The main associated risk factors for the development of GBC include, obesity, chronic asymptomatic cholelithiasis, female gender, smoking, chronic infections of the gallbladder (especially salmonella), diabetes and certain medications such as methyldopa, OCPs, and isoniazid. They are also known to be associated with gallbladder polyps, pancreaticobiliary duct abnormalities, congenital biliary cysts, and carcinogen exposure (Lazcano-Ponce et al., 2001).

GBC is indeed a cancer type with layers of disparities associated with it. The disease prevalence and incidence is extremely high in Latin America and Asia, and comparatively high in some European countries such as Hungary and Poland. GBC incidence in the United States, however, is low. Chile accounts for the highest incidence and mortality rates of GBC in the world, with a higher death rate in women (15.6 per 100,000) compared to men (7.0 per 100,000) (Andia et al., 2008). It is also the leading cause of death in these women, overtaking breast, lung and cervical cancers. The southern region of Chile exhibits a higher incidence and mortality of the disease where there is a large population of the Mapuche (Amerindian) tribe, known to have the highest incidence of GBC. Interestingly, this geographic area of Chile has poor access to medical and surgical facilities attributable to extreme poverty in this part of the country (Andia et al., 2008). Worldwide, incidence of the disease has been shown to increase with age and is found to be two to six times higher in women than in men (Singh et al., 2004a). Interestingly, patients with GBC in India are usually younger ones as compared to their counterparts in the west who are typically in the 5th and 6th decade of their age at the time of presentation with this disease. It is important to note that in India, almost 80% patients with GBC do also have gallstones and its presence increases the vulnerability of the GB to mucosal injury. The incidence of GBC is out of proportion to the prevalence of gallstones in India. On the other hand, in the United States, it is one of the few cancers that has a low incidence in the African Americans while the disease has the highest incidence rates in Hispanics, Alaskan Native (AN) and American Indians (AI) and association of gallstones with GBC is not so much common as compared to India (Henley et al., 2015).

Often, patients with GBC present with severe, persistent abdominal pain that can be diffuse or localized to the right upper quadrant. More often than not, however, the disease is "masked" thereby presenting with no clinical symptoms and as aforementioned, detected incidentally on surgery for gallstone disease. Patients may also present with complaints of weight loss and anorexia - a sign of late stage disease. On examination, they may present with jaundice, abdominal pain in the right upper quadrant with or without associated tenderness. A non-tender palpable mass in the same region (a tenderness is more indicative of gallstone disease), periumbilical lymphadenopathy, and palpable left supraclavicular lymph nodes are also found to be associated with advanced GBC. Nausea, vomiting and abdominal bloating are some of the other vague accompanying symptoms. Prior

to the advent of radiographic techniques in diagnosing GBC, the preoperative rate for detecting the disease was 10-15%. Use of abdominal ultrasound and cross sectional imaging with CT/MRI, have become the mainstay diagnostic tools for detecting GBC. Endoscopic ultrasound (EUS) may also be employed in detecting pre-cancerous lesions. Gallbladder polyps ≥2 cm in size, or a calcified rim of the gallbladder termed "porcelain gallbladder" are indications for surgical resection as these are pre-malignant conditions. However, it is important to note that porcelain gallbladder is no longer thought to be as strongly associated with GBC as previously reported, but this belief continues to be propagated as dogma in the medical literature and textbooks. Several large studies from the United States (articles attached) have shown little to no association between porcelain gallbladder and GBC (Stephen and Berger, 2001; Khan et al., 2011). However, it is important to note that the population in the United States is very different from that of the previous studies from other countries which likely included a large demographic of Amerindians and reported a very high incident of GBC in patients with porcelain gallbladder.

Geographical variability in the incidence of GBC is often found to be associated with the prevalence of gallstone disease in the same population. However, the presence of gallstones alone does not appear to cause cancer in the gallbladder, as populations that have high incidence of gallstones do not necessarily have high incidence of GBC. Chronic infection with Salmonella typhi is another recognized risk factor for GBC (Singh et al., 2004b). A research group from Varanasi, India, in particular, has not only shown the association of chronic infection with gallbladder cancer but also has demonstrated underlying mechanism as to how infection of Salmonella species can help promote transformation of normal gallbladder cells (Sharma et al., 2007; Scanu et a., 2015). Additionally, socioeconomic status and genetic elements that impede access to early detection and surgical procedures such as cholecystectomy are thought to be contributory to poor outcome. In addition to varied geographical incidences, some other factors such as the differences in age, race and sex also contribute to the occurrence of the disease. Figure 1 briefly describes some characteristic features of GBC.

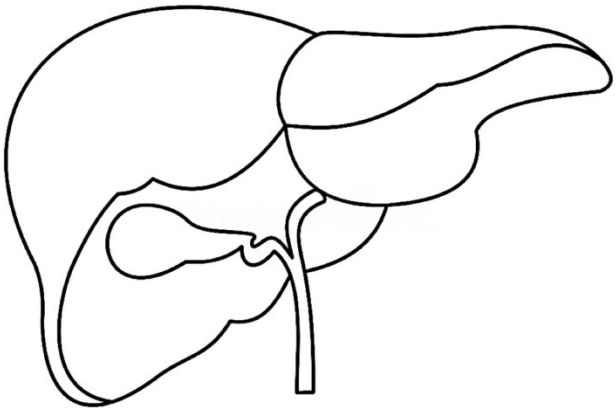

Figure 1: Gallbladder cancer: basic features, risk factors, and therapeutic options.

- Risk Factor: gallstones, gallbladder polyps, chronic cholecystitis, Chronic typhoid carrier state, obesity, diabetes
- Females are at more at risk than their male counterparts
- Typically presents as an incidental finding following cholecystectomy (localized stage) or with abdominal pain (advanced stage)
- Majority of them are adenocarcinoma
- Level 1 evidence for adjuvant chemotherapy: Capecitabine
- Palliative 1st line chemotherapy: Gemcitabine

- No 2nd line palliative chemotherapy with a demonstrated survival benefit over active symptom control
- Median overall survival: ~12 months

Molecular pathogenesis

Late 70s and early 80s was an exciting time in biomedical research for cancer researchers who invested their efforts in establishing a link between cancer and heredity. The role of mutated genes in cancer biology was beginning to be discovered and explored in various types of cancers. Mechanistic insight into discovering the molecular basis for the development of GBC in the western world started very early on and in full force. Reports implicating the role of genetic/hereditary predisposition can be traced back to as early as the 80s (Trajber et al., 1982; Weiss et al., 1984). Karyotyping techniques first were used by Hecht and his colleagues in 1983 to demonstrate the presence of a high level of aneuploidy in the form of missing or extra chromosomes and/or chromosomal rearrangement in a tissue specimen obtained from a Papago Indian woman with GBC in the United States (Hecht et al., 1983). This study reported the presence of double minute chromosomes as well as homogenously stained regions on chromosomes in those cancer cells. A decade later, another group explored the correlation between the DNA content and the cytological and histological features in the tumor cells obtained from the patients with GBC (Roa et al., 1993). These reports were the among the first ones to have used molecular techniques to decipher the molecular/genetic basis for GBC. The following year, a small but an important study conducted by Japanese researchers, demonstrated mutations in the p53 gene in almost one-third of the patients with GBC. They used PCR-single strand conformation polymorphism (SSCP) for the very first time to discover and establish their findings (Takagi et al., 1994). The next several years brought about an increase in the number of published studies mostly from the Japanese investigators. These studies involved discovering various mutations in K-ras (Watanabe et al., 1994; Hanada et al, 1996; Matsubara et al., 1996; Tomono et al., 1996; Saetta et al., 1996; Iwase et al., 1997., Tanno et al., 1998) and p53 genes (Hanada et al, 1996 ; Ajiki et al., 1996; Fujii et al., 1996; Hanada et al., 1997; Jonas et al., 1997; Yokoyama et al., 1998) in a significant percentage of patients presenting with GBC.

By late 1990s, many studies were able to detect the mutations and/or the altered expression of tumor suppressor genes and oncogenes in the patients with GBC. However, most of them lacked mechanistic explanations for their findings. In 1999, a study was published discussing the molecular mechanisms underlying tumorigenesis of gallbladder carcinomas, the first of its kind (Wistuba et al., 1999). The researchers pointed out that while nearly all of the gallbladder carcinomas evolved from dysplasia and carcinoma in situ, role of pre-cancerous lesions such as gallbladder adenomas in the pathogenesis of GBC was still controversial. They further analyzed DNA obtained from micro-dissected tissue samples of pyloric and intestinal-type gallbladder adenomas to compare the molecular abnormalities found in adenomas with the ones seen in carcinomas. Tissue samples derived from carcinoma samples displayed TP53 mutations alongside mutations in both the K- as well as N-ras genes. They also demonstrated loss of heterozygosity (LOH) at chromosomal regions-5q22 APC-MCC region, 9p21-CDKN2a, TP53, RB, and DCC. These genetic aberrations have been known to be associated with and play important

role in tumor initiation and progression in several other cancer types. Since these mutations/molecular changes were not exhibited by adenoma samples, the researchers concluded that adenomatous changes were not similar to those seen in conditions of dysplasia, carcinoma in situ, and/or invasive carcinoma of the gallbladder. Similarly, it was shown that in 25% of adenomas, there was a mutation in the K-ras oncogene at codons 12 and 61, suggesting strongly that these adenomas were not forerunners of invasive GBC (Wistuba et al., 1999). Later in 2001, a Japanese study confirmed this hypothesis by analyzing beta-catenin protein expression and mutation in the corresponding genes. Beta catenin is a regulator of cell-cell adhesion and intranuclear transcription in gallbladder carcinogenesis. The authors also observed that the gallbladder adenomas, in comparison to carcinomas, showed a remarkably high expression of beta-catenin protein in both the cytoplasm as well as the nucleus ($P < 0.05$ and $P < 0.001$ respectively). 62.5% of adenomas, while a mere 4.8% of carcinomas demonstrated exon 3 mutations in the beta-catenin gene. It was further demonstrated that the adenoma to carcinoma sequence was a minor pathway in the pathogenesis of GBC using beta-catenin as a molecular marker) (Yanagisawa et al., 2001).

Besides K-ras and p53, role of other cancer associated proteins were also explored in the pathogenesis of GBC. For example, two mucin proteins MUC1 and MUC2 expressions were examined both at mRNA as well as at protein level in carcinoma (55 cases), dysplasia (20 cases) and non-dysplastic epithelia of the gallbladder, in a Japanese study (Yamato et al., 1999). Based on their observation, the authors suggested that MUC1 expression indicated histological dedifferentiation, increased proliferation and invasion. MUC2 expression, on the other hand, showed a correlation to decreased proliferation with reflection of some differentiation into goblet cells. It was further suggested that expression of MUC1 in a dysplastic gallbladder reflected the potential to developing into a malignancy (Yamato et al., 1999). In the same year, another study from North Korea revealed the role of molecular alterations in the carcinogenesis of gallbladder. They analyzed 32 carcinomatous cases and 11 dysplastic cases for LOH and microsatellite instability (MSI) using 17 microsatellite markers, on chromosomal 3p, 5q, 8p, 9p, 13q, 17p, and 18q sites (Chang et al., 1999). LOH on 5q indicated a premature transformation to carcinogenesis while presence of LOH on chromosomal sites 3p and 9p showed an association to the advancement of GBC. Late event of the disease was likely to be due to presence of LOH on chromosomal sites of 13q and 18q. They observed that LOH on 17p was present not only in dysplastic disease but also found to be higher in the different stages of tumor development. The authors further demonstrated that while accumulation of LOH was associated with carcinogenesis of the gallbladder, role of MI was not of much significance (Chang et al., 1999). A subsequent Japanese study then aimed to investigate the various genetic changes associated with gallbladder carcinogenesis and thus implied that the presence of LOH on chromosome 17p led to the progression of GBC at a fairly early stage, while presence of LOH on 18q and 9p played a crucial role in further advancement of the disease (Hidaka et al., 1999). Another study from Japan emphasizing on the etiologic context of pancreaticobiliary maljunction, a unique yet an important factor associated with GBC in Japanese patients, hypothesized that hyperplastic epithelium played an important role in carcinogenesis. Based

on their findings, they believed that development of neoplasia in a gallbladder with this anatomic anomaly, advanced through the processes of hyperproliferation and genetic variations (Obara et al., 1999).

While most of the early research in Japan was primarily focused on genetic alterations in GBC, a group of medical researchers in Varanasi, a city in India located on the banks of river Ganges where incidence of GBC is very high, started exploring association of various etiologic agents and their role in the pathogenesis of this cancer. They were first research group from India, which demonstrated the presence of Salmonella typhi in gallbladder cancer tissues as compared to control subjects and established the etiopathogenic role of chronic typhoid carriage in gallbladder carcinogenesis (Nath et al., 1997). Another important study from this group in Varanasi, India, demonstrated significantly higher level of heavy metals such as cadmium, chromium, and lead in the patients of GBC than in those with gall stones (Shukla et al., 1998). Role of these well-known chemical carcinogens was further corroborated by the presence of dangerously high concentrations of these metals in drinking water in this geographic location of India. Interestingly, more than a decade later a comparative study using resected gallbladder samples obtained from India and Japan confirmed that Indian patients with GBC had significantly higher level of chromium, lead, arsenic and zinc compared to their Japanese counterparts (Chhabra et al., 2012). This finding indicates towards a need for more focused study to explore the role of heavy metals derived genetic aberrations and relevant signaling pathways so as to develop targeted therapeutics.

By the end of 1990s, it was widely believed that abnormalities in TP53 (17p13) and p16(Ink4)/CDKN2 (9p21-22) loci were regularly encountered and frequently associated with early pathogenesis of the neoplastic disease of the gallbladder. Mutations in the K-ras genes appeared to be a rare neoplastic event, the exceptions of which included anomaly associated GBC. The sequence of dysplasia to carcinoma (CIS) was believed to be a major route for the development of this disease in gallbladders with associated anomalies (Wistuba et al., 1999). In a small but significant study that examined the role of genetic variation in tumorigenesis and as to how these variations were associated with the clinicopathological features of GBC using multiple markers to study MSI and LOH, authors observed that there was one genetic pathway dependent on the MSI pathway and another on LOH pathway in GBC [31]. Interestingly, metastasis to the lymph nodes was not seen in patients with MSI- positive tumors. An inverse relationship was also found between the presence of LOH and MSI in GBC (Yoshida et al., 2000). Soon, a Korean study demonstrated that development of dysplastic and/or subsequent malignant features in GBC was associated with the presence of multiple LOH. Malignant changes from dysplastic gallbladder was associated with alterations in K-ras, p53 and p16 genes, while role of MSI is limited in the development of GBC (Kim et al., 2001). However, a follow up report from Chile which is another geographic area almost endemic for GBC, emphasized the role of MSI in GBC (Roa et al., 2005). This group observed MSI in equal proportions in early as well as late stages of GBC. They also demonstrated that MSI was seen in premalignant lesions and indicated that inactivation of hMLH1, hMSH2, and hMSH6, genes

were responsible for mismatch repair, occurred early in gallbladder carcinogenesis.

In the meantime, contradicting reports on the role of MSI in GBC kept emerging from different parts of the world. A Greek study in 2006 asserted the role of MSI in GBC was minor (Saetta et al., 2001). However, it is vital to make a note of the differences in the results which could be due to a varied selection of markers required to study MSI. It is also imperative to note the role of different mechanisms due to varied etiologies in a diverse patient population, as a possibility. Availability of newer and more effective molecular methods in the early years of the 21st century made it possible for gallbladder researchers to conduct more studies using sophisticated analyses to detect molecular alterations in gallbladder carcinogenesis. A Japanese study using allelotyping analysis demonstrated that GBC associated with anomalous junction of pancreaticobiliary duct (AJPBD) exhibited a high frequency of loss of alleles along with the presence of two new regions, believed to harbor genes with putative tumor suppressor function (Nakayama et al., 2001). In the same year, a group of investigators from Johns Hopkins Medical Institute in the USA developed their interest in studying this intriguing disease and used genome-wide allelotyping analysis, a sophisticated global analytical technique, to reveal multiple sites of allelic loss in GBC (Wistuba et al., 2001). According to this study, frequent allele losses were seen in 21 chromosomal regions, thereby being suggestive of the inactivation of the supposed tumor suppressor genes in the pathogenesis of GBC. This study was the first of its kind, that too from west, where GBC is a rare disease, to provide a global estimate of the extent to which genetic changes were involved in GBC development and to have the potential to identify new tumor suppressor genes and thus identifying numerous new markers in translational research. Another similar study followed up in the the same year from Europe, also demonstrated that alterations in the p53 gene had a likely role in the new pathway of gallbladder carcinogenesis (Saetta et al., 2001).

One of the most significant studies reporting other aberrated molecular lesions (besides p53 and K-ras) in GBC resulted from a collaborative effort between Chile and US based researchers. Presence of a tumor suppressor gene at chromosomal location 3p14.2 containing the fragile histidine triad (FHIT) was shown to be involved in the pathogenesis of this disease (Wistuba et al., 2002). Much later in 2009, another US based study, demonstrated that the loss of FHIT and a fragile gene product WWOX expression were part of an earlier events underlying pathogenesis of GBC (Bloomston et al., 2009). The same year, a group based in India analyzed LOH and MSI in FHIT gene (Priya et al., 2009). This group of investigators from India also looked for the expression of the p53 gene in GBC, Chronic Cholecystitis (CC), Xanthogranulomatous Cholecystitis (XGC) and the normal gallbladder to establish the role of the FHIT gene in the gallbladder cancer using microsatellite markers such as D3S1217, D3S1300, D3S1313, D3S1600, and D3S2757. This study reported 17.5% of MSI and 27.5% of LOH in the GBC cases from India. Significant differences were also noted in LOH between GBC and CC (p=0.002) and GBC and normal GB (p=0.02). Their results suggested that CC acts as a pre-invasive lesion in the pathogenesis of GBC (Priya et al., 2009. It is important to note that some researchers believe that XGC, an uncommon variant of CC, is a precursor lesion of GBC. This was the first report to

study and compare genomic instability in GBC versus different variants of CC and normal gallbladder from any Asian country. However, an earlier report in 2001 had studied XGC at molecular level and confirmed that while etiopathologic factors of XGC could have a correlation to cancer, XGC might not be an immediate cause of cancer (Takada et al., 2002).

In a study conducted in Italy, expression of the p53 gene as well as microsatellite instability (MSI) status were studied in tumor tissues obtained from 71 patients with GBC. Examination of all neoplasms were conducted using IHC staining for hMSH2, hMLH1, p53 proteins and markers of GI differentiation along with a microsatellite analysis at mononucleotide locus BAT-26. The authors showed that although MSI was not a key factor in GBC development, alteration in the p53 gene played a critical role in a large percent of cases with the exclusion of mucinous and squamous GBC (Sessa et al., 2003). Concurrently, a small study at Johns Hopkins Medical Institute, found hypermethylated regions at various promoter sites of tumor suppressor genes that contributed to the formation of the tumor as well as its progression inside a GB that was chronically inflamed. Since this study included a large cohort of specimens obtained from two different populations, Chile and the United States, plain disparities in the patterns of methylation between the gallbladders obtained from these two countries led to the hypothesis that these differences indicated a varied and distinctive biology associated with this disease around the world (House et al., 2003). In a subsequent Chilean study, mutations in mitochondrial DNA (mtDNA) was observed in GBC which suggested that mtDNA be additionally investigated in patients from different geographical regions thus including it in a list of molecular biomarkers for early detection of GBC (Tang et al., 2004).

Same year, investigators at Johns Hopkins demonstrated that GBC had a unique pattern of abnormal gene methylation in a study which encompassed the most inclusive methylation profiling. The finding of methylation in gallbladders with CC (and without cancer) in healthy individuals suggested that this was an important phenomenon in the early pathogenesis of GBC (Takahashi et al., 2004). Subsequently, a group from Chile observed that the high frequency of methylation of promoter areas for CDKN2A (p16), FHIT, APC, and CDH1 genes led to the inactivation of genes responsible for tumor suppression and control of cellular proliferation in the carcinogenesis mechanism (Roa et al., 2006). Another Chilean study then confirmed by epigenetic inactivation that abnormal methylation in the promoter regions of tumor suppressor genes such as DUTT1 (3p12), FHIT (3p14.2), BLU, RASSF1A, SEMA3B and hMLH1 (3p21.3) on chromosome 3p was a frequent occurrence (Riquelme et al., 2007).

A small study from Greece reported mutations in the B-raf gene for the first time, which is an important component of Ras signaling pathway (RAS/RAF/MEK/ERK). They reported that almost one third of all patients with GBC also presented with mutations in the B-raf gene (Saetta et al., 2004). It is imperative to note that the Ras pathway is an organized, downstream pathway in which Raf proteins are activated in a Ras dependent manner. Considering the fact that Ras signaling pathway could be activated by mutations at various levels including B-raf, it was an important finding of re-emphasizing the role of Ras pathway in those GBC cases where no mutation was detected in the K-ras

oncogene (Saetta et al., 2004). Same year, our group demonstrated that almost one third of all the patients with GBC has mutations in codon 12 of the K-ras oncogene (Singh et al., 2004b). While this study was the first of its kind from India to use methods to detect changes at a molecular level in an oncogene, it also demonstrated that detection of such molecular aberration in an oncogene could be performed on a small number of tumor cells retrieved from fine-needle aspirates. Additionally, together with cytological examination, this work showed a possibility to help making a definitive diagnosis in patients with other risk factors, at least in a sub-set of patients harboring K-ras mutations. Besides, this work also suggested the role of chronic inflammation in the etiology of gallbladder carcinogenesis (Singh et al., 2004b).

In the dawn of 21st century, with growing realization of cancer being a much more complex disease, than it was thought to be earlier, cancer researchers working on GBC initiated pathway-mechanism oriented studies in various parts of the world. One such study conducted in Japan cited factors such as homozygous deletions, promoter and LOH hypermethylations and multiple LOH to be the major mechanisms involved in inactivation of the p16 gene in GBC (Tadokoro et al., 2007). Another such study to understand geographical differences in genetic changes involved in GBC between Japan and Hungary, was conducted in a collaborative effort by collecting specimens obtained from patients living in these two countries. A considerable difference between the two populations was attributed to the presence of high MSI seen in Japanese patients as opposed to just one high MSI patient in Hungary. This further affirmed the notion that geographic variation could play a significant role in the process of gallbladder carcinogenesis at mechanistic level (Nagahashi et al., 2008).

Investigators in the United States relying on serial analysis of gene expression (SAGE) created cDNA libraries from the tumor tissues of stage matched GBC patients of Native American, Caucasian and Hispanic/Latino descent, along with the tissues from normal gallbladder. RT-qPCR analysis was performed on the microdissected epithelia extracted from both GBC as well as their corresponding non-neoplastic mucosae. To further understand the complexity of the disease, immunohistochemistry on the GBCs was done in a complex tissue microarray format. The hallmark of this study was using SAGE to make an impartial assessment of the transcriptome in GBC, and identifying a new positive prognostic marker-expression of connective tissue growth factor (CTGF). GBC and non-neoplastic mucosae from the gallbladder have SAGE libraries which are available publicly at the Cancer Genome Anatomy Project that would help facilitate the research needed for this deadly cancer (Alvarez et al., 2008). In another small attempt to identify metastasis-associated proteins in GBC, a Chinese study demonstrated that overexpression of chloride intracellular channel 1 (CLIC1) encouraged cell motility and inundation of GBC-SD18L (cell line with a low potential for metastasis) *in vitro*. It was interesting to note that inhibition of CLIC1 expression by RNAi significantly decreased the cell motility as well as the invasiveness of GBC-SD18H (a cell line with a high potential for metastasis). This study thus demonstrated that CLIC1 could play a crucial role in GBC metastasis, and was hence identified to be a regulator of metastasis in GBC (Wang et al., 2009). An overview of some such key studies reporting mutational spectrum in GBC is provided in Table 1. In another such

interesting study from India, investigators identified E-cadherin (CDH1) genomic instability and demonstrated a correlation between their findings with CDH1 protein expression in conditions of GBC and other non-neoplastic conditions of CC, XGC, as well as with normal GB, to define its role in GBC carcinogenesis. More importantly this study provided solid evidence as to how CC acts as precursor lesion to GBC (Priya et al., 2010).

Table 1. Important mutations during various stages of gallbladder cancer development.

Gene and Reference	Adenoma (%)	Dysplasia (%)	Carcinoma (%)
K-Ras			
Hanada et al., 1999	17	15	38
Watanabe et al., 1999	0		19
Kim et al., 2001	0	0	20
Wistua et al., 1999	25		
Singh et al., 2004			38
Li et al., 2014			8
Beta-Catenin			
Yanagisawa et al., 2001	63		5
Rashid et al., 2001	57		9
Chang et al., 2002	58	0	0
Kumari et al., 2014			4
EGFR/ErbB2/ErbB3			
Nakazawa et al., 2005			16
Leone et al., 2006			15
Pignochino et al., 2010			10
Li et al., 2014			37
P161NK4A			
Kim et al., 2001	0	0	31
Ueki et al., 2004			62
TP53			
Wistua et al., 1999	0		
Kim et al., 2001	0	0	36
Li et al., 2014			47
Kumari et al., 2014			18
Noguchietal., 2017			64

Abovementioned studies to identify genetic alterations in the GBC were based on conventional sequencing methods such as Sanger sequencing, which had its own limitations. In the recent past, altered genetic pathways involved in human cancer are being studied using next-generation sequencing (NGS) technologies which has really helped in understanding the disease mechanism. NGS has numerous advantages when compared to the conventional sequencing methods in that it is a highly throughput method. It permits a large number of parallel sequencing simultaneously in multiple genomic regions of many samples to identify any associated mutations in the same run sequence. Another crucial benefit to using NGS is the reduction in the turnaround analysis time

during routine tumor sequencing, which ultimately will lead to short clinical reporting time. In addition, the amount of RNA/DNA required for analysis is very low in comparison to traditional methods. Genomic aberrations such as single/multiple nucleotide variants, copy number variations (CNVs), fusion transcripts, and small and large insertions and deletions can be detected simultaneously with high sensitivity and rate of accuracy. The highlight of NGS is the high rate of sensitivity and quantitative evaluation of the mutated allele that is significantly better than Sanger sequencing (2-10% vs 15-25%).

A group of investigators from China were the first to take advantage of the newly available NGS technique to identify new mutations in GBC (Li et al., 2014). Here, they studied 57 pairs of tumor-normal tissues and subjected them to a combination of exome as well as ultra deep targeted sequencing of cancer- related genes [54]. They found that there was an increase in C>T/G>A transition in GBC. This identification of somatic mutation framework of GBC was done in a systematic manner, discovering GBC driver genes in the C>T/G>A transition along with studying a pathway centered around ErbB signaling. They also pointed out that genetic mutations in the ErbB pathway was a poor prognostic indicator of the disease. This study also went on to suggest that patients exhibiting genetic mutations in the ErbB pathway could potentially benefit from therapies already available or in the process of development (Li et al., 2014). In a similar kind of study using NGS techniques, a Japanese group analyzed 29 tissues obtained from Japanese patients with GBC, using an integration of whole-exome and transcriptome sequencing techniques to uncover molecular variations and thus, prospective therapeutic targets (Nakamura et al., 2015). It was suggested in this study that APOBEC-mediated somatic mutational signature, associated with APOBEC3B expression and a higher number of mutations, selectively contributed to GBC initiation and progression. The Japanese authors also reaffirmed the findings in the previous study (Nakamura et al., 2015) and suggested that activation of the EGFR family of genes (EGFR, ErbB2, ErbB3) was seen frequently in GBC and could be clinical important as they could be used as potential targets.

A recent study by one of the authors (BM) of this article, determined the presence of 184 non synonymous somatic mutations and 60 rare germline mutations in SMAD family member 4 (SMAD4), lysine methyltransferase 2C (KMT2C) and TP53 genes using ultra deep sequencing across 409 cancer related genes in GBC patients of Northern India (Yadav et al., 2017). Of note, somatic mutations of high significance within 9 novel genes in GBC were identified. The results in study hinted at the presence and significance of rare, inherited germline mutations in the genes of the DNA-repair pathway in addition to acquired somatic mutations in the carcinogenesis of GBC. A high throughput sequencing based analysis of the mutation profile in GBC was carried out for the very first time by an Indian group from the northern part of the country. This study was different in the sense that they restricted the study to regions of common genetic aberrations so as to take advantage of targeting sequencing approaches and covering rare mutations high confidence. Cancer initiating as well as progressing events were tagged with biological pathways associated with 409 cancer genes which were analyzed in the study (Yadav et al., 2017). The somatic mutation spectrum in this study was found to be dominated by substitution of the C>T/G>A

transition, consistent with other GBC sequencing studies (Li et al., 2014; Nakamura et al., 2015). This study from India (Yadav et al., 2017) holds promises for its possible role in future precision medicine practice in GBC, as it suggests that both the somatic mutation profile as well as the information regarding various germline mutations could be beneficial for understanding the disease pathology as well as for developing novel therapeutic strategies.

Genetic polymorphism and risk of GBC

It is believed that majority of the cancers are derived from single somatic cells along with the cells derived by the rapid division of those parent cells. These new cells acquire various genetic or epigenetic changes within them that lead to altered genetic and thus to phenotypic variants. However, with the advent of the Human Genome Project in early 2000s and published evidences started supporting the theory that common genetic variations play important role in determining the range of individual susceptibility to neoplastic diseases within a particular population. It was thought that they may result, among others, from the differences in the metabolism of environmental carcinogens and mechanisms of DNA repair and the kind and rate of metabolism is genetically determined by polymorphic enzyme coding genes participating in the process of xenobiotic transformation. A large number of enzymes involved in the reaction of oxidation or conjugation of exo-endogenous xenobioties have shown genetic polymorphism. Gene variability may alter the expression or enzymatic activity of coded enzymes. First such study in GBC was conducted in Japan in which authors investigated the correlation between the genetic polymorphisms in cytochrome P4501A1 (CYP1A1) gene and the risk of development of GBC. The frequency and relationship between the CYP1A1 genetic polymorphisms and the development of GBC was closely examined in order to determine the differences in susceptibility to developing the disease. They demonstrated that females with genotypes C and/or Ile/Val in CYP1A1 gene were potentially more susceptible to developing GBC (Tsuchiya et al., 2002). Next was our own study that was performed on a large cohort of 153 patients with GBC from North India (Singh et al., 2004a). We reported that the frequency of the X- allele of apolipoprotein B (apoB) was significantly increased in GBC patients irrespective of the presence or absence of gallstones (GS). The odds ratio was found to be 2.3 and 1.7 respectively. Apolipoprotein B (apoB) is a gene that is widely known to be associated with alteration in serum lipid levels and susceptibility to gallstone (GS) disease. By the abovementioned findings, we suggested that a polymorphism in the apoB-XbaI gene results in increased susceptibility to GBC in a favorable environment (Singh et al., 2004a).

With time many other similar studies started appearing looking for polymorphic variants associated with risk of developing GBC in endemic areas. In 2006, a Chinese study investigated the potential risk associated with polymorphisms in the Cytochrome P450 17alpha-hydroxylases-C-(17,20)-lyase (CYP17) enzyme (involved in synthesis of sex hormones) in GBC. It was found that CYP17 MspA1 polymorphism was associated with an exaggerated increase in GBC development and was also contributory to the development of biliary stones among the overweight and diabetic individuals. This finding led to the study of an intertwining relationship between the genetic and hormonal risk factors associated with gallbladder disease

(Hou et al., 2006). Same year, a study conducted by one of the authors (BM) in India reported an association between glutathione S-transferase (GST) polymorphic variants (GSTT1, GSTM1, GSTP1, and GSTM3) with risk of gallbladder cancer (Pandey et al., 2006). These GSTs are a family of detoxifying enzymes and are thus intricately involved in the metabolism of many known and potential carcinogens. Various allelic variants of these polymorphic GSTs show deformed enzymatic activity and thus are reckoned to increase cancer risk and susceptibility. This was first study to show a correlation between Val allele of GSTP1 and an increased risk of GBC in the Indian population (Pandey et al., 2006). Following year, same group including one of authors of this article [BM] went on to show that a polymorphic variant of N-acetyl transferase2 (NAT2) which is also known as slow acetylator phenotype, influenced the susceptibility of GBC (Pandey et al., 2007). That same year a study from China demonstrated that Ser326Cys polymorphism in human oxoguanine glycosylase 1 (hOGG1), a gene involved in the excision repair mechanism for damaged DNA, has an association with GBC (Jiao et al., 2007). Another case- control study was undertaken in Japan in order to examine and determine the relationship between the various genetic polymorphisms of genetic polymorphisms of cytochrome P450 1A1 (CYP1A1), glutathione S-transferase class mu (GSTM1), and tumor protein p53 (TP53) genes, and GBC risk, observed that the Val allele of CYP1A1 Ile462Val and the Pro allele of TP53 Arg72Pro polymorphisms aided in a heightened incidence of GBC among Japanese women and men, respectively (Tsuchiya et al., 2007). Another collaborative study demonstrated that Val allele of cytochrome P4501A1 (CYP1A1) gene contributes to the development of GBC in women of two different populations of Japan and Hungary (Kimura et al., 2008).

Last decade has seen a growing body of data to demonstrate an association between various polymorphic variants of many cancers associated genes with risk of GBC. Majority of these studies came from researchers including one of the authors of this article (BM) from North India. Some of the important ones are those that report a strong link between genetic variants of signaling molecules such as Tumor Necrosis Factor alpha (TNFA) and Interleukin 6 (IL6) (Vishnoi et al., 2007), A-204C of cholesterol 7alpha-hydroxylase (CYP7A1) (Srivastava et al., 2008a), Cholecystokinin receptor A gene polymorphism (Srivastava et al., 2008b), Interleukin-1 gene polymorphism (Vishnoi et al., 2008), Single nucleotide polymorphism in ABCG8 transporter gene (Srivastava et al., 2009), toll-like receptor gene polymorphisms (Srivastava et al., 2010a), and Caspase-8 gene polymorphisms-- genes that regulate apoptosis in cancer cells (Srivastava et al., 2010b). In another significant study on larger sample size obtained from northern India from the same group led by one of us (BM), strong association of a haplotype of tumor suppressor gene DCC, Grs2229080-Ars4078288-Crs7504990-Ars714 was reported to confer high risk of GBC in northern Indian population (Rai et al., 2013a). In a follow up genome wide association study (GWAS) using same patients' cohort, this group also identified that some polymorphic variants of Phospholipase C epsilon 1 (PLCE1) gene which is known to play a critical role in both formation and progression of esophageal and gastric cancers and PSCA gene which plays an important role in inhibition of cell proliferation and/or inhibition of cell death. This study showed that these polymorphic variants confer susceptibility to GBC through gallstone-

mediated inflammatory pathway and in a gender specific manner respectively (Sharma et al., 2013; Rai et al., 2013b).

One of the authors (BM) of this article, recently published a GWAS study which to our knowledge is the largest study worldwide as it comprised of 523 GBC patients and 274 controls all from northern India (Yadav et al., 2018). This study reaffirms the fact that the link between genetic polymorphism and disease risk may be very specific with regards to directional effect and histology/ethnicity by demonstrating that genetic variants on TERT-CLPTM1L and 8q24.21 loci affect GBC prognosis and predisposition. In order to describe the inter-relationship between genetic variants and GBC susceptibility in populations at risk, the study stressed on the need to perform complete gene sequencing of the 5p15.33 and 8q24.21 loci. However, it is important to note that in spite of huge amount of efforts involved in these studies to study genetic risk factors, it is still unclear that which genetic pathways are actually important in order to make a person susceptible living in a geographically prone area for GBC such as North India. All these genes demonstrated above which have been shown to be associated with GBC as risk factor belong to different signaling pathways, which further complicates the issue of making a coherent picture of all these genetic susceptibility factors. Many more studies have been published in recent past from different parts of the world showing one or other type of polymorphic variants of different genes involves in metabolism, signal transduction, drug-metabolizing enzymes related pathways, however, most of them are actually adding more complexity to the existing mechanistic insights about GBC.

Interestingly, genetic aberrations in genes associated with inflammation which is an important aspect of gallbladder carcinogenesis has not been much explored in these GWAS studies. FOXM1, NF-kB, STAT3, Wnt/β- Catenin, HIF-1α, NRF2, androgen and estrogen receptors are some examples of the tumor suppressive as well as oncogenic transcription factors, the interaction between which, results in chronic inflammation of the gallbladder. The chronic inflammation may be either due to stones or a long standing infection of the organ. Studies conducted in various clinical models, pre-clinical samples as well as cell lines, show that numerous products obtained from natural resources namely, polyphenols, alter the expression and activity of many transcription factors in various tumor models (Agrawal et al., 2015). Additional information regarding alterations in the aforementioned genes and their regulator pathways could help design a new and improved form of therapy combining these natural products along with the standard currently approved chemotherapeutic regimen. The combined therapy has been considered promising and the hope is to be able to not only treat the inflammation and cancer but also prevent the two conditions.

Targeted Therapy in GBC

While there were a multitude of studies by mid 2000s that demonstrated that overexpression of tyrosine kinase growth factor receptors such as ErbB-2, epidermal growth factor receptor (EGFR), and Met could potentially lead to the formation of solid tumors, there were a group of investigators in Japan who studied biliary tract carcinomas. The investigators, with the intention of assessing novel chemotherapies, focused on these receptors in GBC and demonstrated overexpression of the

tyrosine kinase receptor proteins by IHC in tumor tissues obtained from 89 GBC patients. Another technique, fluorescence in situ hybridization was used for gene amplification in tumor tissues that stained positive. There was an overexpression of the ErbB-2 and the EGFR genes in 15.7 and 8.1% of GBC cases with GBC gene amplification of 79%. This study suggested the new adjuvant chemotherapies could be used in GBC in which ErbB-2 and EGFR are overexpressed (Nakazawa et al., 2005). Later, an italian study observed that in a subgroup of patients with GBC, somatic mutations of the EGFR gene in the tyrosine kinase domain exhibited cell signals maintaining proliferation and survival. Further these investigators suggested a small molecule inhibitor of EGFR for treatment (Leone et al., 2006). That same year, a Chinese study reported that p53 - vascular endothelial growth factor (VEGF), a marker for angiogenesis) pathway potentially played a role in regulating tumor angiogenesis in GBC (Tian et al., 2006). This study suggested that analyzing the expressions of the p53 as well as the VEGF genes could be useful in predicting the tumor vascularity in GBC and targeting advanced tumors with anti-VEGF inhibitors could be a good therapeutic strategy.

In order to better understand the underlying mechanisms of tumor development and progression and to improve the prognosis of GBC patients, an Austrian study observed an increased expression in the HER2/neu gene which clinically and statistically correlated with advanced disease (Puhalla et al., 2007). In various cancer types, it has been shown that p110alpha catalytic subunit of phosphatidylinositol 3-kinase (PI3K), encoded by the PIK3CA gene harbor somatic mutations. Exons 9 and 20 which encode the helical and kinase domains of the p110alpha are the major hotspots for clusters of genetic mutations. Another group based in Switzerland also reported that somatic mutations in the PIK3CA gene resulted in recurrent activations of the PI3K/AKT pathway in a very small sub-set of carcinomas of the gallbladder (Riener et al., 2008).

Having realized the limitations in treatment options other than surgical resection (which was an unfavorable prognostic marker), a group of surgeons in Japan identified cellular targets that were GBC specific and which could potentially be used as a therapeutic approach for the disease. They identified a cell cycle-related gene, topoisomerase IIalpha (TOPO IIalpha) as one of the highly upregulated gene in GBC tissue which they further confirmed as a potential chemotherapeutic target, because those cells strongly positive for TOPO IIalpha had shown increased sensitivity against etoposide, as well as doxorubicin and idarubicin (Washiro et al., 2008). In the subsequent year, another Japanese group demonstrated genomic instability due to amplification in Myc oncogene which resulted in specific amplification of EGFR and/or ERBB2 in GBC (Ooi et al., 2009). Another *in-vitro* study used a combination of a histone deacetylase inhibitor (SAHA) with repression of EZH2 by siRNA treatment which revealed an increased sensitivity of the GBC cells to SAHA as opposed to the normal cells. This was then considered to be indicative of the efficacy of the new anti- cancer agent (Yamaguchi et al., 2010).

In a significant study to evaluate the response rate by Response Evaluation Criteria in Solid Tumors (RECIST) of targeted therapy in biliary cancers which included GBC, eligible patients (10 patients with GBC) were treated with bevacizumab (a VEGF inhibitor) and erlotinib (EGFR inhibitor) in combination with chemotherapy. Using a

combination chemotherapy of bevacizumab with erlotinib resulted in clinical activity with infrequent grade 3 and 4 adverse effects in advanced GBC cases. Preliminary molecular analysis showed that presence of a mutation in the k-ras oncogene altered the efficacy of Erlotinib. Though a small study in sample size (only 10 cases of GBC), results clearly warrant a larger study in future on a bigger cohort of GBC patients to investigate the efficacious performance of Bevacizumab and erlotinib when used together, as an alternative treatment option for patients with advanced GBC (Lubner et al., 2010). In an independent but encouraging small case report, the idea of using EGFR-tyrosine kinase inhibitor (TKI) in combination with conventional chemotherapy was tested to treat gallbladder cancer in the US based hospital (Mody et al., 2010). In this case report, a patient with stage IV GBC was shown to have complete and prolonged response, a not so common observation in GBC, to oral EGFR-TKI plus chemotherapy regimen. Of note, this study mentions that this rare response to the treatment for this patient with GBC was due to an absence of a mutation in the EGFR gene. This discovery should again reiterate the need for clinical trials using EGFR-TKIs to treat GBC. This study also suggest that future clinical trials should not always consider the mutation status of the EGFR gene as inclusion criteria (Mody et al., 2010).

In an Italian study, investigators analyzed mutations, amplifications and over-expression of EGFR, HER2, and their molecular transducers in biliary tract cancers so that they could explore possibilities of combining standard therapies with or without molecular targeting (Pignochino et al., 2010). They reported that EGFR was expressed in ~38% of patients with GBC. Activated forms of cancer relevant signaling proteins such as p-MAPK and p-AKT were also highly expressed in little less than 46% of GBC, hinting at an activation of the EGFR pathway. With the aid of genomic amplification, about 10% of GBCs showed an overexpression of HER2 gene. They went on to conduct a preclinical in-vitro study using TGBC1-TKB, a GBC cell line (deleted on PTEN and negative for Her2 expression) for testing the efficacy of the drugs either alone or in combination with gemcitabine, targeting the aforementioned molecules. This study also demonstrated that the HER2 and EGFR pathways could pose to be potential therapeutic targets for biliary tract cancers (BTCs). The combination of gemcitabine with Gefitinib and Lapatinib (both of which are reversible selective inhibitors of the tyrosine kinase domain of EGFR and drugs known to target HER2 and EGFR pathways) appeared to have encouraging results and thus warrants for further clinical studies for testing the expression levels and mutations in these signaling molecules in future clinical studies (Pignochino et al., 2010).

While developing strategies to target GBC molecularly, it is important to understand the subtle changes at the molecular/genetic level between GBC and other related cancers. Although they are histologically similar, they are anatomically different in terms of the origin of the tumor. For example, cholangiocarcinomas arises from within the liver parenchyma, peri-hilar regions, or the distal biliary tree, and these tumors are collectively known as BTCs. Although these tumors share an anatomic origin in the biliary system, they have very different disease patterns, molecular profiles and also respond differently to various therapies. Historically, GBC has a tendency to initially be sensitive to chemotherapy but the survival rate is much shorter when compared to cholangiocarcinoma (Eckel et al., 2007).

Traditionally, treatment of BTCs with cytotoxic chemotherapy have not taken into account the anatomic origin of tumor or its molecular profile. However, in light of emerging molecular techniques, such as cost effective sequencing platforms, it will be worthwhile to have a detailed molecular genetic profiling of patients with GBC before considering a regimen of targeted therapy for GBC.

Increasingly, molecular tests to detect changes at genetic level are being applied regularly (in developed countries where advance health facilities are available to common people) to aid in the forming of therapeutic decisions in cancer treatment. Routine testing such as genetic amplification of HER2/NEU, and/or genetic mutations involving EGFR and Kras are done in clinics so as to determine treatment benefit with targeted specific anti-cancer therapies (McDermott et al., 2009). Patients in which molecular genetic profiling has been conducted that indicate a potential benefit with EGFR inhibitors and other molecularly targeted drugs, BRAF inhibitors are also being tested at the earliest phases of the disease (Brower T, 2010). Discovering patterns of genetic changes within GBC is very critical in order to not only gain insight into the disease biology but also to bring out about improvised treatment options, especially in light of emerging and established studies showing that tumor genetics determine drug sensitivity. In another study carried out at Massachusetts General Hospital in the US, almost 13% of patients with GBC were identified with activating mutations in PIK3CA (Deshpande et al., 2011). Activating mutations in the PIK3CA pathways has dual advantages in it being helpful for cancer diagnosis as well as discovering new targets for therapies such as PI3 kinase inhibitors.

Most recently, in a large whole-exome and targeted gene sequencing based study, a Chinese chohort of GBC patients, several recurrent mutations in erbB pathway were identified (Li et al., 2014). In this study, it was observed that genes like TP53, K-ras, and ErbB3 had a significant frequency of non-silent mutations of 47.1%, 7.8%, and 11.8% respectively with a false discovery rate (FDR) of <0.0.5. Furthermore, it was also discovered that the ErbB signaling pathway including EGFR, ErbB2, ErbB3 and ErbB4 along with their downstream genes, affected 36.8% of GBC samples, making it the most extensively mutated pathway. Mutations in the ErbB pathway genes were also found to be associated with a dismal prognosis in GBC patients, thus suggesting that targeted therapies, that are presently in development or already in use in clinics, could be of major benefit (Li et al., 2014). Role of ErbB pathway in GBC was further confirmed in few other independent studies from different cohorts of patients. In one of such study, retrospectively conducted in the patients with GBC in the United States, HER2/neu blockade was found to be a promising treatment strategy to treat GBC patients who have gene amplification (Javle et al., 2015). In another significant study conducted in Japan, in a large cohort of biliary tract cancers, EGFR family genes such as ErbB2 and ErbB3 were found to be activated while PTEN and TSC1 genes were found to be inactivated, in GBC. Frequent genetic variations in the TP53 and RB cell cycle modules were also reported in GBC (Nakamura et al., 2015).

An interesting study from India using integrated genomic and proteomic analysis provides a compelling evidence that ERBB2 is an important therapeutic target under neo-adjuvant or adjuvant settings for treatment of patients with GBC. In addition, this study also shows that presence of K-

ras mutations may preclude patients with GBC to respond to anti-EGFR treatment, similar as to how a clinical algorithm is often used to opt for anti-EGFR treatment in patients with colorectal cancer (Iyer et al. 2018). Most recently, in a collaborative study between investigators from India and the US, PIM1 kinase has been demonstrated to promote cell proliferation of gallbladder cancer cells via inhibition of PRAS40 (Subbannayya et al., 2019). It is important to note that several small molecule inhibitors of PIM1 kinase have been developed, some of which are currently being tested for their efficacy in clinical trials for several types of cancers. Currently, some of the authors of this article (CPG, ALL and MKS) are investigating the role of Myc-Aurora kinase signaling in the gallbladder cancer and as to how this signaling axis can be therapeutically targeted (unpublished work).

Conclusion

Recent studies employ large cohorts of GBC to emphasize the differences between the various GBC subtypes at a basic genetic/molecular level. Functional studies and clinical trials only including patients with specific subtypes of GBC, and stratifying them based on their genetic drivers, were the next steps to accomplishing these goals. For instance, use of inhibitors of the EGFR were to be examined only in the patients with GBC carrying mutations/genetic amplifications of the EGFR gene. Constructing a precise approach to genomic medicine for the treatment of this disease uses a combination of biological studies, drug research and development, technological advancements in genomics as well as research that determines health outcomes. Based on the genomic information as summarized in Table 2, several clinical trials are currently underway to examine the efficacy of targeted therapies in patients. This, in the long run, is going to help build a staircase to developing a targeted therapy for GBC. Based on the outcome of these studies, we should be able to offer effective therapies based on their genomic profiles to the patients with deadly GBC in the future.

Table 2: Ongoing clinical trials using targeted therapies in gallbladder cancer.

Drug(s)	Target	In combination with	Phase	Clinical Trials Identifier
Cetuximab, Gefitinib Trastuzumab, Lapatinib, Everolimus Sorafenib Crizotinib	EGFR, HER2, HER2, EGFR mTOR RAF kinase, VEGFR-2/PDGFR-beta ALK and ROS1	Gemcitabine, Oxaliplatin (GEM OX)	II	NCT02836847
Sorafenib	RAF kinase, VEGFR-2/PDGFR-beta	Gemcitabine and Cisplatin P	II	NCT00919061
Guadecitabine Durvalumab	DNA methyltransferase Programmed cell death-1 ligand 1	-	I	NCT03257761

Sorafenib	RAF kinase, VEGFR-2/PDGFR-beta	Gemcitabine, Oxaliplatin (GEMOX)	II	NCT00955721
Regorafenib	Receptor tyrosine kinase, VEGFR2	-	II	NCT02053376
Pazopanib	Receptor tyrosine kinases including VEGFR-1, VEGFR 2, VEGFR-3, PDGFR-α and –β, FGFR -1 and -3	Gemcitabine	II	NCT01855724
Ramucirumab	VEGFR2		II	NCT02520141
Lapatinib	HER2, EGFR	-	II	NCT00107536
Durvalumab, Tremelimumab	Programmed cell death-1 ligand 1 CTLA-4	Gemcitabine, Cisplatin	II	NCT03473574
Pembrolizumab	Programmed cell death protein 1	Gemcitabine, Cisplatin	II	NCT03260712

In patients with advanced stages of unresectable GBC, treatment options are limited because of no so well defined molecular targets. Complete surgical resection remains the only curative modality to treat patients with GBC, offering benefit only for patients with localized disease. As evident from the past studies on GBC, most research efforts have focused on identifying genetic mutations, which did not provide much insight into signaling molecules that could be targeted therapeutically. GBC still continues to have a poor prognosis worldwide. It is widely accepted that cancer is primarily a signaling disease. Therefore, one strategy to identify functional therapeutic targets in GBC is to get deeper insights into major signaling mechanisms underlying initiation and progression of gallbladder cancer, which will have potential to be used in clinical decision-making as well as conducive to the design of personalized cancer treatments. Given the ethnic, geographic, and gender disparities associated with gallbladder cancer, lack of funding and resources to initiate prospective studies using cutting edge omics studies to decipher signaling events underlying gallbladder cancer is a major impediment. In recent years, with identification of few molecular targets in GBC such as ERBB2, ERBB3, and MYC amplifications, it is becoming apparent that development of effective therapies will be benefitted by focusing on personalized therapy such as by identification and targeting of genetic mutations specific to an individual patient. Investigation of epidermal growth factor receptors (EGFR) such as Cetuximab and Erlotinib in a clinical trial for GBC patients' cohorts will in turn help decide whether or not such targeted therapeutics can be recommended as standard-of-care.

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Conflict of interest

The authors declare no conflict of interest.

Authors' contributions

This invited review article was conceptualized and designed by BM and MKS. Review of literature and other data was collected by CPG, ALL, and MKS. Article was written, reviewed, and edited by MRK and MKS.

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