Chapter II

Review of Literature

2.1 Epidemiology of GBC

2.1.1 Global trends of GBC incidence

The epidemiology of GBC shows a unique distribution pattern worldwide [**Figure 2.1**]. Several studies reported that incidence and mortality rates of GBC are very high in North India, Chile, Eastern Europe, Latin America, and other Asian countries [1-2]. Chronic cholecystitis occurs due to GSD, which is one of the major causes of GBC in specific regions. In Latin American countries such as Chile, Bolivia, and Mexico, the incidence rate of GBC is very high [3]. Chile contributes to the highest GBC-related incidence and mortality rate in both sexes [4]. In Chile, a large group of GBC patients have cholelithiasis, and chronic cholecystitis is the most common clinical manifestation associated with GBC in Chilean populations [5]. GBC is considered to be the second cause of cancer-related death in Chilean women [2]. GBC accounts for 4% of all gastrointestinal cancers in both sexes, 6% in females and 3% in males. Globally, the incidence and mortality age-standardized rates (ASR) are higher in female populations (2.4 and 1.8 per 100,000 females per year, respectively, versus 2.2 and 1.6 per 100,000 men per year), except in some Asian countries such as the Republic of Korea and Japan, where males have the highest ASR values [3]. Due to demographic changes, the global burden of GBC is expected to rise by more than 75% (165,600 new cases and 130,400 new deaths) by 2040 [6].

Asia is the most high-risk continent for GBC incidence. The North Indian and Pakistani females and Korean males have increased the frequency of GBC cases. The Korean population has the highest GBC incidence rates in Asia [7]. The highest rate of GBC occurrences is reported in Chile followed by the North-Indian region. The geographical regions associated with greater than average GBC incidence rates mainly focus on regions of Asia and Latin America [7]. In addition to geographical or regional variations, there are distinct ethnic disparities. The incidence of GBC is low among black individuals residing in the United States (US). However, the frequency of GBC cases is extremely high among American Indian, Alaska Native, and Hispanic individuals. Recent studies reported that Hispanic women of US origin have 3-5 times higher GBC incidence rates compared to non-Hispanic women [8-9]. This distinct geographical and ethnic variation in GBC incidence rates suggests that there is a strong interplay between environmental and genetic etiological factors [7]**.**

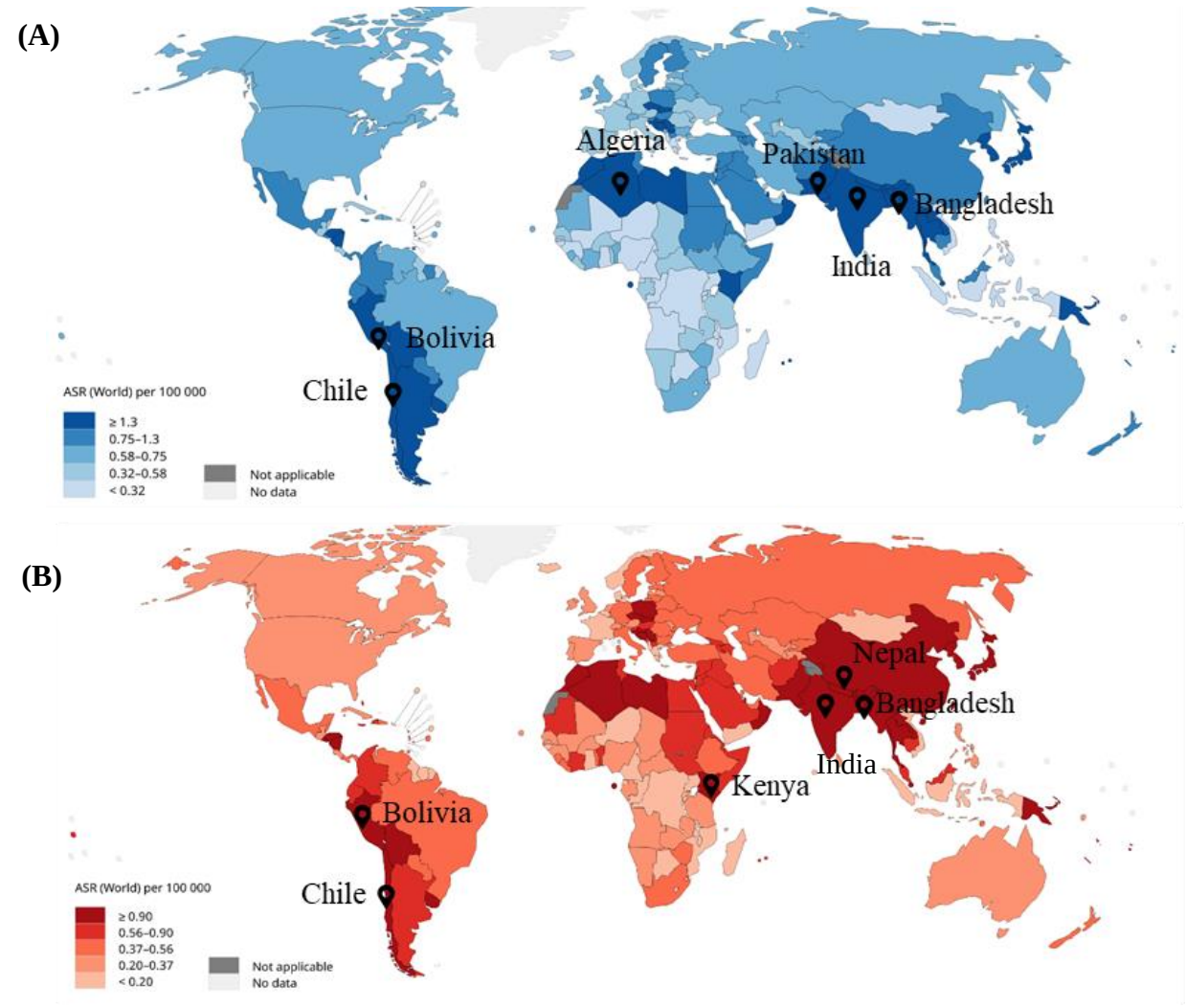

Figure 2.1: Estimated age-standardized global incidence **(A)** and mortality **(B)** rates of GBC patients in both sexes. (The countries with the highest GBC incidence and mortality rates are highlighted). Figure generated from GLOBOCAN 2020 (https://gco.iarc.fr/).

2.1.2 GBC incidence in India

India represents about 10% of the global burden of GBC. The ASR for GBC in women in northern and northeastern India is 11.8/100,000 and 17.1/100,000, respectively. The incidence rates of GBC are extraordinarily high in the North, North-East, and Central regions of India [**Figure 2.2**]. ASR applies weights based on a standard population's age distribution instead of using a reference population's age distribution. The GBC incidence in North and North East India is comparable with the regions with the highest GBC incidence in the world such as Chile,

Bolivia, Japan, and Poland [10]. Women in Delhi and Bhopal have GBC incidence rates as high as 6.6 and 5.2, respectively. It is the fourth most common cancer in Delhi (after cervix, breast, and ovary), and the most frequent gastrointestinal cancer in women. Similarly, in Bhopal, GBC (incidence 5.2) was the fifth most common cancer in women (after cervix, breast, mouth, and ovary, and the most common gastrointestinal cancer in women. In Jammu, GBC is the third most common cancer in women and the leading cause of malignant obstructive jaundice in Lucknow. Moreover, many GBC cases have been reported from various health centers and hospitals in North East India [11]. The evidence that the Indian population has a higher risk of developing GBC is reflected in the higher GBC incidence in those who have migrated to other regions of the world. Studies reported that GBC was eight times more common in Indian migrant women in Fiji as compared to native Fijian women. In the UK, higher mortality rates were observed in GBC patients of Indian origin than in the UK-born populations in both sexes [12]. Even today, the pathogenesis of GBC is poorly understood in India. It appears to be a multi-step pathological process where the genetic and epigenetic alterations accumulate as a result of host and environmental factors. These cumulative genetic changes eventually result in mutagenesis, leading to GBC development.

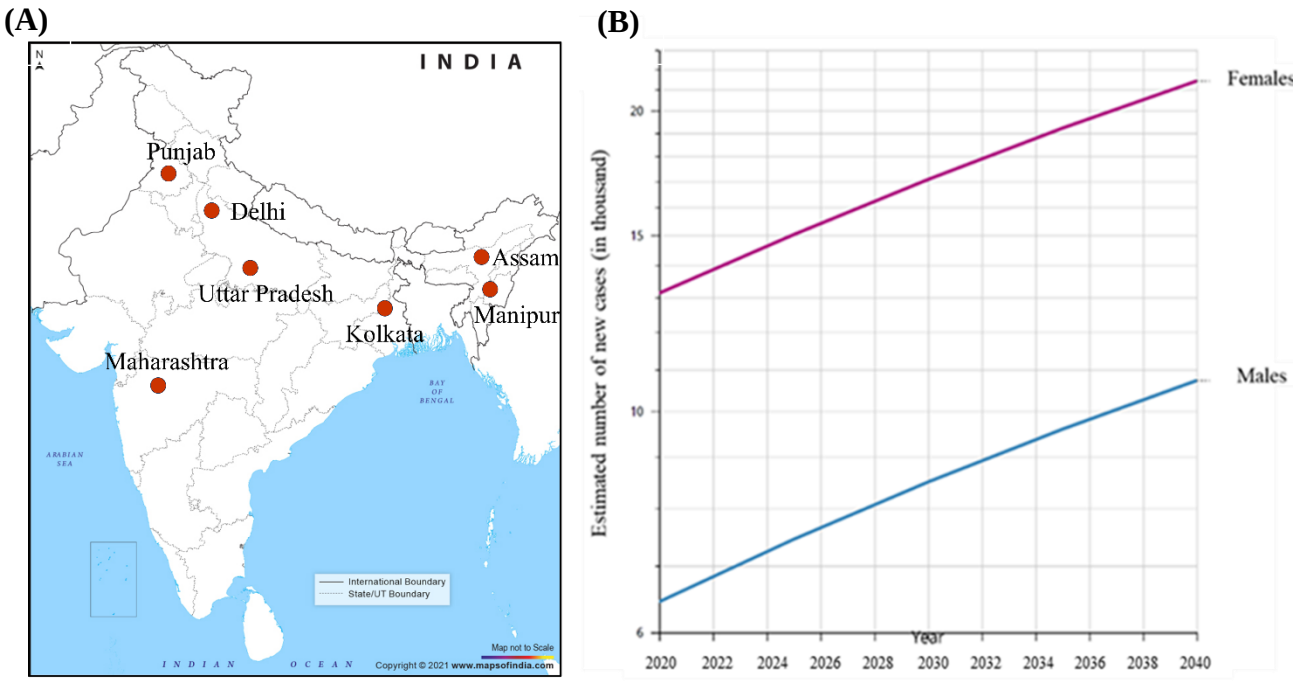

Figure 2.2: Incidence of GBC in India. **(A)** States with high incidence rates are indicated in red dots. **(B)** The gender-specific burden of GBC cases in India from 2020-2040.

2.1.3 Incidence of GBC in Assam

Assam has the highest incidence of GBC in both men and women. The incidence was highest in the Kamrup urban district (Age-adjusted rates (AAR) of 7.9 in males and 16.2 in women), then Cachar district (AAR of 5.6 in men and 11.9 in women). AAR involves calculating the cancer incidence or mortality rates for a specific population while adjusting for differences in age distribution by applying weights to age-specific rates. In the North-East, it has been found that women are more likely than men to develop gall bladder cancer [13]. Several risk factors are found to be associated with GBC in this region such as the presence of pesticides, aromatic hydrocarbons, nitrosamines, nitrates, and nitrites [14]. Moreover, contamination of heavy metals, mainly iron, lead, and cadmium in Brahmaputra, Ganga, and Pachin rivers and groundwater [15-16], and the possible presence of adulterants in edible mustard oil used for cooking in the region are the possible factors that induce gallbladder carcinogenesis in this region [17]. Chronic cholecystitis and bile acid degradation caused by bacterial infection are some of the predominant factors for GBC development. These risk factors correlate significantly with *Salmonella typhi* infection which is quite prevalent in several parts of NE-India [18]. However; the exact cause of the high incidence rate of GBC patients in NE India is still unknown.

2.2 Gallstone disease and its role in GBC pathogenesis

Gallstones (GS) are one of the major risk factors leading to GBC. In the Western population, cholesterol GS represents a large percentage (80-90%) of all GSD cases and is thought to be an important promoting factor in GBC progression. However, there is no significant information available to determine whether pigment or cholesterol stones operate differently as promoters of GBC [18]. Mechanistically, the development of GBC from GSD is thought to result from the continual irritation of the GB epithelium, which causes inflammation and increased cell regeneration [18-19]. Chronic inflammation eventually activates GBC progression through pathological sequence- metaplasia→dysplasia→in situ carcinoma, in which cells undergo genomic and epigenomic changes that may lead to invasive GBC within 5-15 years [**Figure 2.3**]. A small number of cholelithiasis patients develop GBC [19], and about twenty percent of patients with GBC have no prior history of cholelithiasis. However, in high-risk regions, this percentage is potentially greater and increases significantly with age [20-21].

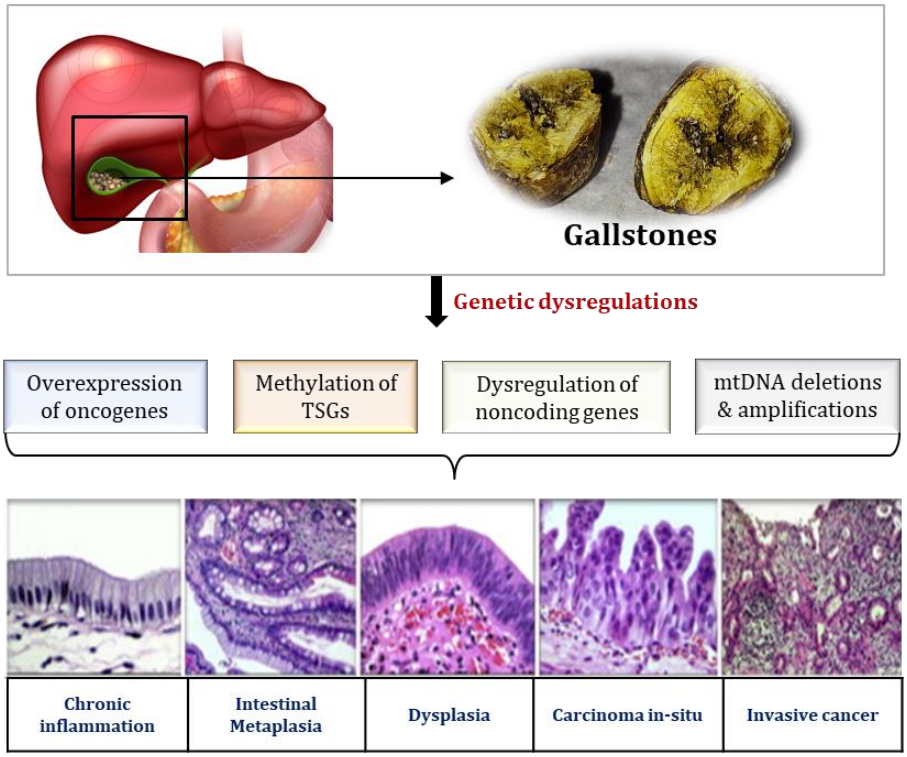

Figure 2.3: Mechanistic diagram showing the progression of GSD to GBC through different pathological spectra resulting in multiple genomic changes/dysregulations and finally leading to invasive GBC progression (Reproduced from [20]).

GS are classified into two categories based on their chemical makeup and macroscopic appearance (cholesterol and pigment GS), each with its etiology. The production of cholesterol GS is caused due to dysregulation in biliary cholesterol homeostasis, which disrupts the physical-chemical balance of cholesterol solubility in bile [22-23]. A study of 43,141 Swedish twin pairs with GSD revealed that genetics accounts for 25% of the risk [24]. GS heritability is greater than 50% among Hispanics with Native American ancestry [25]. Multiple lithogenic gene variations have been linked to gallstone formation, indicating that the genes involved are very diverse [26]. Studies reported that Elevated blood triglycerides (TG) levels might be the most significant independent predictor of GBC risk in GSD patients, while insulin resistance is an important GBC risk factor in patients without GS. More notably, it was found that a significant increase in blood TG levels acts as a possible diagnostic or prognostic biomarker of GBC with GSD [27]. Although, the gallstone disease condition has historically been linked to GBC risk, however; the exact etiology of GBC development from GSD is still unknown.

2.3 Molecular pathogenesis of GBC

Based on morphological, genetic, and molecular data, two unique independent biological mechanisms were hypothesized that result in GBC: (1) a dysplasia-carcinoma sequence deriving from metaplastic epithelium, and (2) an adenoma-carcinoma sequence [28-29]. The histological analysis of GBC samples demonstrated GBC progression in a stepwise manner from hyperplasia-metaplasia-dysplasia or gallbladder adenomas. Kozuka et al., (1982) reported that adenomas larger than 12 mm were prone to GBC development [30]. According to Roa et al., (2006), the predominant carcinogenic pathway in the development of GBC is the dysplasia-carcinoma sequence, and malignant transformation of a gallbladder adenoma is relatively rare [31]. Moreover, as per the findings by Watanabe et al., (1999) distinct carcinogenetic profiles may emerge, given that *KRAS* mutations are associated with the adenoma-carcinoma pathway, while p53 mutations are linked to the dysplasia-carcinoma pathway [32]. The dysplasia-carcinoma sequence continues to be the most widely recognized pathological spectrum for the molecular pathogenesis of GBC, given the generally low incidence of gallbladder adenomas. The pathogenesis of GBC has been linked to various genomic changes including activation of oncogenes, methylation /mutation in tumor suppressor genes (TSGs), microsatellite instability (MSI), and loss of heterozygosity (LoH) [32-33].

2.3.1 Genetic mutations

The precise genetic alteration that contributes to the development of GBC is still poorly understood. Several genetic alterations are found to be associated with GBC pathogenesis including oncogene activation, tumor suppressor gene inhibition, microsatellite instability, and methylation of gene promoter regions. In GBC, over 1281 gene mutations have been identified and [28,34] several genes have been reported to be dysregulated in GBC [**Table 2.1**]. For instance, early molecular events are thought to include p53 mutation, cyclooxygenase-2 (*COX2*) overexpression, mitochondrial DNA mutations, and hypermethylation of promotors in tumor suppressor genes, with later events including the mutation of the fragile histidine triad (*FHIT*) and cyclin-dependent kinase inhibitor 2A (*CDKN2A*) tumor suppressor genes as well as loss of regions on chromosomes 9, 18, and 22. In GBC, dysplasia further results in the overexpression of p16 [34]. Like many other cancers, *Kras* and *TP53* are the most well-known genes associated with GBC.

A few studies performed Genome-Wide Association Studies (GWAS) to investigate the mutational spectra in GBC. In a recent study, the link between genetic variation in the ABCB1 and ABCB4 genes and GBC risk has been reported for the first time in the Indian GBC population [35]. A study by Kumari et.al performed mutational profiling of paraffin-embedded GBC tissues in the Indian population and identified *TP53, CTNNB1, PIK3CA*, and *KRAS* as important mutational signatures in GBC [36]. GWAS in biliary tract cancer has reported the *APOBEC* gene and ErbB signaling pathway as crucial mutational signatures in GBC [37].

2.3.2 Epigenetic modifications in GBC

Epigenetic regulation is a well-defined mechanism in gene expression that occurs either through transcriptional repression or activation [38-39]. The most extensively investigated epigenetic mechanism, DNA methylation, occurs at the C-5 position of the cytosine in the cytosine-guanine dinucleotide (CpG) and is implicated in various biological activities [39-40]. Approximately 1% of the human genome consists of CpG islands, and 60% of the promoter region contains CpG islands [38]. GBC is specifically influenced by epigenetic regulation. The methylation patterns of the tumor suppressor genes *p16, APC, MGMT, hMLH1, RARbeta2*, and *p73* have been found in 72% of GBCs and 28% of chronic cholecystitis, and such methylation patterns are rare in normal tissue [41]. The methylation rates were compared between GBC patients from Chile and the United States in accordance with the variation in global prevalence and it was found that there is a significant difference in the methylation of *APC* (42% versus 13%) and *p73* (14% versus 40%), indicating a distinct geographical variation [42]. It is believed that the degree of methylation level increases throughout the chronic cholecystitis progression via the development of metaplasia [34,43].

2.3.3 Microsatellite Instability and Loss of Heterozygosity in GBC

The insertion or deletion of repeat units, which alters the number of sequence repeats in microsatellite markers, is known as MSI [44]. MSI plays an important role in carcinogenesis through inactivation of several genes. Studies on the role of MSI found that the pathogenesis and prognosis of GBC are associated with GBC [45]. In 10% of GBC patients, studies have identified MSI-H markers D13S317, FES/FPS, and F13A01 [46]. There have been reports of high MSI in E-cadherin (*CDH1*) (67%), and *FHIT* (17.5%) from Indian GBC patients. However,

the contribution of microsatellite instability (MSI) to the carcinogenesis of the gallbladder is still poorly understood [47-48]. Apart from MSI, LoH is one of the most commonly occurring genetic aberrations in the cancer genome. Several mechanisms can lead to LoH such as the deletion of an allele, duplication of a chromosome, or chromosomal region. LoH is associated with several tumor suppressor genes in GBC mainly including *RB, VHL, RASSFIA, FHIT, APC, PRLTS, FEZ1, DBCCR1, WWOX,* and *FRA16D* [34].

Table 2.1: List of reported key genes dysregulated in GBC patients.

Gene	Gene name	Role	Expression	Ref
KRAS	Kirsten rat sarcoma virus	Oncogene	Upregulation	[49-50]
EGFR	Epidermal growth factor receptor		Upregulation	[34]
ERBB2	Erythroblastic leukemia viral oncogene homolog 2		Upregulation	[51-52]
TP53	Tumor protein 53	Tumor suppressor	Upregulation	[53]
P16 (CDKN2A)	Cyclin-dependent kinase inhibitor 2A (CDKN2A)		Downregulation	[54]
VHL	Von Hippel-Lindau		Downregulation	[55]
FHIT	Fragile histidine triad		Downregulation	[49,54]
Rb	Retinoblastoma		Downregulation	[54]
THBS1	Thrombospondin-1	Cell-cell and cell-matrix interactions	Upregulation	[49]
COX-2	Cyclooxygenase-2	Angiogenic factors	Upregulation	[49]
VEGF-A	Vascular endothelial growth factor		Upregulation	[56]
CDK2	Cyclin E	Cell cycle regulators	Upregulation	[54]
CCND1	Cyclin D1		Upregulation	[54]
P27Kip1 (CDKN1B)	Cyclin-dependent kinase inhibitor 1B		Downregulation	[49,54]
Caspases	Cysteine-dependent, aspartate-specific peptidase	Regulators of pro and anti-apoptotic proteins	Upregulation	[48]
Bcl-2	B-cell lymphoma 2		Upregulation	[49]
	Cadherins	Cell-cell adhesions	Upregulation	[57]
MUC1	Mucin-1	Regulator of intracellular signal transduction	Upregulation	[58]

2.4 Regulation of noncoding RNAs in GBC pathogenesis

For decades, medical research primarily focused on only tiny protein-coding regions of the genome. The human genome project revealed that only 2% of our genome codes for proteins, and the remaining 98% of the human genome comprises noncoding parts. Later on, the ENCODE project revealed that the noncoding portion of the genome copied into thousands of RNA molecules that not only regulate fundamental biological processes such as growth, development, and organ function but also play a critical role in the pathogenesis of human disease, particularly cancer [59]. Noncoding RNAs (ncRNAs) are identified as the key regulators of gene expression. Dysregulation of ncRNA expression is the key characteristic of cancers. The ncRNAs are highly cell, tissue, and cancer-specific, thus transcriptome profiling of ncRNAs is important for the identification of cancer-specific diagnostic, prognostic, and therapeutic biomarkers [60]. The ncRNAs are classified into short (19-31 nucleotides), mid (20-200 nucleotides), and long (>200 nucleotides) groups based on their length. The microRNAs (miRNA), which fall under the short ncRNA group, have received the most attention in cancer, whereas; long noncoding RNAs (lncRNAs) are emerging as the key regulators involved in cancer development and progression. miRNAs mediate post-transcriptional gene regulation through translational repression, mRNA degradation, or methylation. However, lncRNAs regulate gene expression via their interaction domains for mRNAs, miRNAs, and proteins [61].

2.4.1 Role of miRNA regulation in GBC

miRNA was initially identified as the product of the lin-4 gene in Caenorhabditis elegans in 1993 [62-63]. Depending on the degree of complementarity, miRNA develops post-transcriptional regulation through mRNA cleavage or translation repression, which depends on the complementarity degree of miRNA-mRNA. Perfect matching leads to mRNA cleavage, whereas incomplete pairing results in gene suppression [64]. Numerous studies have established the association of microRNAs in cancer-related biological processes and pathways, including proliferation, differentiation, apoptosis, metabolism, invasion, metastasis, and treatment resistance. Studies also demonstrated that the dysregulation of miRNAs is directly associated with the pathological etiology of cancer [65].

Dysregulation of a single or a few miRNAs was found to have a significant impact on the overall expression pattern of several hundred mRNAs which drives malignant transformation. miR15 and miR16 at 13q14 are the first human disease-related miRNAs that were first characterized in chronic lymphocytic leukemia [66-67]. Furthermore, individuals with diffuse large B-cell lymphoma have significantly higher levels of tumor-associated miRNAs, indicating the potential role of miRNAs in cancer development and pathogenesis [68]. The discovery of miRNAs led to a worldwide research effort to establish their roles in cancer. miRNAs regulate molecular pathways in cancer by targeting various oncogenes and tumor suppressors [69-70]. The discovery of the role of miRNAs in cancer development showed that miRNAs act as critical regulators of important cancer-associated pathways by targeting different oncogenes and tumor suppressor genes. Transcripts that play a role in promoting apoptosis or suppressing cell growth are directly impacted by oncogenic miRNAs. On the other hand, tumor-suppressor miRNAs suppress the expression of oncogenes and/or genes involved in cell differentiation or apoptosis [71].

The first study on miRNA expression profiling in GBC was performed on transgenic BK5.erbB2 mice, in which Kitamura et al., (2012) [72] expressed the murine ErbB2 gene under the promoter of the bovine keratin 5 in the basal layer of epithelial tissues to develop GBC. miRNA expression in GBC tissues differential expression in transgenic mice as compared to wild-type mice. Additionally, the histone deacetylase inhibitor PCI-24781 therapy dramatically restored these aberrant miRNAs. For instance, *miR-21*, *miR-142-3p*, *miR-142-5p*, and *miR-223* were downregulated after PCI-24781 treatment, despite being elevated in GBC tissue. However, PCI-24781 dramatically increased *miR-122*, which was downregulated in GBC, suggesting the chemotherapeutic potential of these miRNAs in GBC pathogenesis [73]. Several studies used large-scale microarray analysis to identify the miRNA expression profiles in GBC tissues and cells. The identified miRNAs show altered expression, with some miRNAs being overexpressed but the majority are found to be downregulated. Further studies supported the role of these miRNAs as either oncogenes or tumor suppressors. Recent studies showed that the levels of circulating miRNA were considerably distinct in GBC patients as compared to healthy volunteers and were linked to tumor clinical features [73-74]. Several other studies on miRNA expression profiling [**Table 2.2**] have identified many significantly altered miRNAs in GBC.

Table 2.2: List of reported altered tumor suppressors and oncogenic miRNA identified in GBC.

	Dysregulated miRNAs	Expression in GBC	Target genes	Clinical role	Ref
Onco-miRNA	*miR-21*	Up	*PTEN*	Cell proliferation, migration, TNM metastasis, and poor progression	[72]
	miR-155	Up	--	Lymph node metastasis, invasion, poor prognosis	[76]
	miR-20a	Up	*SMAD7*	Invasion, metastasis, and prognosis	[75]
	miR-182	Up	*CADM1*	Migration and metastasis	[77]
Tumor suppressors miRNAs	*miR-218-5p*	Down	*BMI-1*	Cell proliferation and metastasis	[78]
	miR-34a	Down	*PNUTS*	Poor prognosis	[79]
	miR-130a	Down	*HOTAIR*	Cell proliferation and invasion	[80]
	miR-135a-5p	Down	*VLDLR*	Histological grade	[81]
	miR-26a	Down	*HMAG2*	Cell proliferation, colony formation, and TNM metastasis	[82]
	miR-146b-5p	Down	*EGFR*	Tumor size, progression, and TNM stage	[83]
	miR-1	Down	*VEGF-A; AXL*	Proliferation and migration	[84]
	miR-145	Down	*AXL*	Proliferation and migration	[84]
	miR-143	Down	--	Lymph node and TNM metastasis	[85]
	miR-122	Down	--	Lymph node metastasis	[85]
	miR-187	Down	--	Lymph node metastasis	[85]

2.4.2 Mechanism of lncRNA regulation

LncRNA is commonly defined as a noncoding RNA molecule that is larger than 200 nucleotides and is not translated into proteins [86]. According to the proximity between neighbouring transcripts, lncRNAs are broadly classified into sense, antisense, bidirectional, intronic, and intergenic [87-88]. Like mRNAs, the lncRNAs are transcribed by RNA polymerase II and undergo post-transcriptional processes including capping, polyadenylation, and splicing [89]. Due to the low levels of expression of lncRNAs and the presence of established kinds of unstable transcripts like the promoter upstream transcripts (PROMPTs), the lncRNAs have traditionally been assumed to be extremely unstable [90]. However, a recent study suggests that only a small percentage of lncRNAs (29%) are unstable with half-lives of 2 hours, whereas, 6% of lncRNAs were found to be highly stable with a half-life of about 12 hours [90]. LncRNAs are found to be

localized in different cellular compartments which include both the nucleus and cytoplasm. Furthermore; the lncRNAs are found to be expressed in a tissue, developmental stage, and disease-specific manner and thus lncRNAs can serve as a potential therapeutic target for diseases including cancer [91-93].

The lncRNAs are associated with diverse biological functions and therefore three general paradigms- guides, scaffolds, and molecular decoys have emerged to broadly classify lncRNA functions [94-95]. For the precise localization/organization of elements at particular genomic loci for genome regulation, lncRNA guides are necessary. LncRNA guides are directed to specific locations in the genome at either cis (near) or trans (remote) sites from their locus of transcription by binding to regulatory or enzymatically active proteins that include transcription factors and chromatin modifiers [94-96]. By serving as pivotal platforms for the transient assembly of various enzyme complexes and other regulatory co-factors, lncRNAs act as dynamic molecular scaffolds and contribute to gene expression regulation. The Telomerase RNA component (TERC) is a classical example of an RNA scaffold that assembles the telomerase complex and maintains the end of the telomere [97-98]. By functioning as a molecular sink, lncRNA decoys primarily serve to restrict the availability of particular regulatory components. LncRNA decoys regulate gene expression by sequestering RNA-binding proteins, transcription factors, microRNAs, catalytic proteins, and pieces of larger modifying complexes, several lncRNAs, including MEG3 and TUG1, have been demonstrated to sequester different microRNA from protein and mRNA targets, altering the translation and degradation of the resultant proteins [99-100].

2.4.3 Potential lncRNA biomarkers identified in GBC

LncRNAs regulate gene expression through transcription, post-transcriptional regulation, or epigenetic mechanisms to play critical roles in cellular processes such as cell proliferation, differentiation, DNA damage response, chromosomal imprinting, etc. [101]. LncRNAs function as both tumor promoters and suppressors, and abnormal lncRNA expression is strongly linked with carcinogenesis and progression [102]. Studies demonstrated that lncRNAs are variably expressed in tumor tissues and serum of GBC patients, making them relevant as predictive and

diagnostic biomarkers. Different lncRNAs have been studied for their potential to have various effects in GBC, where they can act as either tumor suppressor or oncogene [93].

2.4.3.1 Oncogenic lncRNAs in GBC

Studies revealed that the expression of *MALAT1* is highly overexpressed in GBC tissue samples compared with adjacent non-tumor tissues. It was found that the ERK/MAPK signaling pathway was suppressed in GBC tissues and cell lines when *MALAT1* was silenced or knocked down, thus inhibiting the expression of *MALAT1* in GBC and reducing the malignant properties [103]. The lncRNA-*DILC* acts as a tumor suppressor lncRNA in liver cancer stem cells. However, in GBC, DILC was found to act as an oncogene. Lnc-*DILC* stimulates the growth of GBC stem cells by activating the Wnt/-catenin signaling pathway and promotes the development of GBC [104]. Another oncogenic lncRNA-*H19* was found to be significantly overexpressed in GBC tissues as compared to adjacent normal and positively correlated with EMT progression and overall survival of GBC patients. Studies confirmed that H19 contributes to EMT progression in GBC through dysregulation of transcription factor Twist1 [105]. LncRNA- *HOXA* cluster antisense RNA 2 (*HOXA-AS2*) is significantly upregulated in GBC patients and is associated with increased cell proliferation and EMT progression [106]. Recent research has revealed that *SPRY4-IT1* plays a role in the development of several tumor types and acts as an oncogenic regulator in several cancers. According to Yang et al., *SPRY4-IT1* knockdown markedly reduced GBC cell growth, migration, and metastasis. *SPRY4-IT1* is found to be an established lncRNA biomarker in GBC, making it a potential candidate for a new treatment approach [107]. Several malignancies, including colorectal and gastric cancer, have been linked to *CCAT1*. It has been demonstrated that this lncRNA is more abundant in GBC tissues as compared to normal tissues. Furthermore, compared to the early stages of tumors, *CCAT1* is more strongly expressed in the advanced stages of tumors. *CCAT1* expression is associated with a poor prognosis in GBC. Additionally, in vitro research demonstrates that *CCAT1* knockdown reduces S-phase, invasion, and tumor growth in vivo. Mechanistically, *CCAT1* regulates the expression of Bmi1 through competitively sponging *miRNA-218-5p* [78]. A recent study demonstrates that in DOX-resistant GBC cell lines, GBCDRlnc1 overexpression contributes to an increase in autophagy activity inside the cells. Doxorubicin, gemcitabine, and 5-fluorouracil resistance in DOX-resistant GBC

cells was found to be considerably increased by high GBCDRlnc1 levels, indicating that GBCDRlnc1 may be responsible for the chemoresistance shown in GBC cells [108].

2.4.3.2 Tumor suppressive lncRNAs in GBC

Most of the lncRNAs identified in GBC are overexpressed. However certain lncRNAs also experience downregulation. *LET* and *GATA6-AS* are two instances of this, which have been discovered to be downregulated in GBC tissues as opposed to non-tumor tissues. Low LET expression was associated with more advanced tumor stages, higher lymph node invasion, and less differentiated histology. Functionally, *LET* knockdown increases GBC cells' ability to invade and proliferate in anoxic environments. *LET* overexpression, on the other hand, induces apoptosis while decreasing proliferation and tumor growth in vivo. These findings imply that *LET*'s reduced expression may contribute to the development of GBC [109]. The expression of *GATA6-AS* lncRNA was also shown to be significantly downregulated in advanced-stage GBC patients and its overexpression significantly decreases the proliferation and migration in GBC cell lines [110]. It has been shown that lncRNA-*MEG3* is downregulated in GBC tissues as compared to nearby non-cancerous tissue. *MEG3* overexpression dramatically reduced cell proliferation, colony formation, and the ability of GBC cell lines to induce apoptosis. The NF-kB pathway and the large tumor suppressor 2 (*LATS2*) are regulated by MEG3 in a mechanism that promotes EZH2 degradation via ubiquitination [111]. Similarly, lncRNA *GCASPC* is also downregulated in GBC tissues and is associated with larger tumors, advanced disease stages, lower rates of overall survival (OS), and disease-free survival (DFS), among other factors. Ectopic *GCASPC* expression reduced proliferation and significantly increased G1-S arrest in GBC cell lines, whereas cell lines overexpressing *GCASPC* produced smaller tumors in nude mice. These results suggest that high *GCASPC* expression inhibits carcinogenesis in GBC. Mechanistically, *GCASPC* acts by inhibiting pyruvate carboxylase through miR-17-3p regulation [112].

2.4.4 Competitive endogenous RNA (ceRNA) regulation in GBC

miRNA influences hundreds of genes and therefore acts as critical post-transcriptional components in DNA-RNA-protein networks [113]. Recent research on the role of miRNA has produced significant findings that support the ceRNA theory. Two essential concepts in the

ceRNA hypothesis are miRNA and miRNA response element (MRE). miRNA regulates mRNA and lncRNA in the post-transcriptional processes by binding to the MRE of the mRNA as well as lncRNA and pseudogenes [114]. Importantly, each miRNA has a large number of RNA targets, and the majority of RNA molecules contain several MREs, making them targets of various miRNAs. Due to the abundance of targets, it has been proposed that various RNAs compete for the limited pool of miRNAs, thus acting as ceRNAs [115]. A study showed that in addition to miRNA inhibiting the function of mRNA, mRNA can also affect miRNA in the opposite direction. As a result, protein-coding RNA or noncoding RNA functions by competing with the same MRE, serving as the fundamental component of this mechanism. For example, *PTENP1* impacts the expression of *PTEN* mRNA as they share the same MRE. In this case, fewer MRE is "saturated" when *PTENP1* is downregulated, more *PTEN* mRNAs will bind to miRNA, which will ultimately limit *PTEN's* post-transcriptional activity [116]. Several lncRNA and miRNA have been identified that function as ceRNA in GBC pathogenesis and development. For instance, lncRNA-*H19* competitively binds with *miR-342-3p* and regulates the expression of FOXM1 in GBC cells [117]. In GBC tissues, overexpressed lncRNA-*CCAT1* stimulated the growth and invasiveness of GBC cells by competitively "sponging" *miRNA-218-5p,* which in turn elevated the miRNA-218-5p target gene *Bmi1* [78]. Another lncRNA, *HOTAIR* works in part by suppressing *miRNA-130a* by activating *c-Myc*, which acts as an oncogene in several cancers including GBC [83]. In GBC cells, the ceRNA component- *MINCR/miR-26a-5p/EZH2* axis was identified to play a role in cell proliferation, invasion, EMT, and apoptosis [118]. Studies in GBC cells found that lncRNA-*GCASPC* is a target of *miR-17-3p* and that both *GCASPC* and *miR-17-3p* inhibit the pyruvate carboxylase activity by limiting its protein stability [112]. lncRNA-*NEAT1* regulates survivin expression by acting as a ceRNA for *miR-335* in GBC [119]. In GBC, *DGCR5* acts as an oncogenic lncRNA by sponging miR-3619-5p and regulates MEK/ERK1/2 and JNK/p38 MAPK pathways in GBC pathogenesis and development [120]. The above-mentioned experimental studies show that lncRNAs play a significant role in GBC by acting as ceRNA. However, more studies are required to identify novel lncRNA signatures and their associated mechanism that drive GBC pathogenesis.

2.5 Transcriptional regulation: Transcription factors as key regulators in GBC

The human genome encodes over 2000 different transcription factors (TFs), many of which are expressed in a cell type-specific manner regulating the gene expression programs that are involved with a vast array of cellular processes [121]. TFs are proteins with DNA binding domains that interact with RNA polymerase II and other cofactors to control the transcription of a target gene by binding to specific DNA sequences in the promoter and/or enhancer region [122]. TF activity in malignancies can be changed in several direct and indirect ways. Chromosome translocations, gene amplification or deletion, point mutations, and changes in expression are examples of direct mechanisms; indirect mechanisms include non-coding DNA mutations, and epigenetic processes like DNA methylation and histone modification, among others. Due to their crucial role in the development and evolution of cancer as well as in malignant transformation properties such as invasion, metastasis, and chemo-resistance, TFs are considered as mainstay of cancer development [123]. Studies revealed that 20% of oncogenes exhibit functionality as TFs. In the intricate landscape of cancer biology, the constitutive expression of these onco-TFs is imperative for driving cancer-cell proliferation, growth, and invasion. In contrast, tumor-suppressor TFs play a critical role as negative regulators in the cell cycle process, influencing DNA repair and apoptosis. The loss of function in these tumor suppressor TFs leads to the dysregulation of cell division, paving the way for uncontrolled cell proliferation and malignant transformation [124-126]. For instance, *p53* is one of the most studied TF that is regulated by several genes involved in cell cycle regulatory pathways. Studies reported that approximately 50% of cancers have a mutation in the p53 gene, which is considered a crucial event in cancer development [127]. Kruppel-like factor 4 (*KLF4*) lowers the expression of Slug in prostate cancer [128] and controls the expression of E-cadherin in breast cancer cells [129] to prevent metastasis. Similar to this, *KLF4* activation in nasopharyngeal cancer regulates stemness properties, suppresses migration and invasion, and a mesenchymal-epithelial transition (MET) [130]. A recent study discovered genes with paradoxical roles and found that 50% of those genes encoded TFs act both as tumor suppressors and oncogenes in various malignancies [124].

It is evident from several studies that TFs play a key role in the pathogenesis and progression of cancer and therefore can be used as a potential drug target. In GBC, there are only a few studies that demonstrated the role of TFs in GBC pathogenesis. A recent study reported that *TCF4* plays an oncogenic role in the progression of GBC and could serve as a new potential therapeutic biomarker for GBC [131]. GLI2, a TF played an important physiologic role in GBC. In GBC, GLI2 was found to be associated with *PD-L1* expression, fibrosis, and invasion [132]. A recent single-cell transcriptome study on GBC reported IRF8 as a potential TF that might be associated with immunosuppression and metastatic invasion in GBC and therefore IRF8 could be associated with immunotherapy outcomes for metastatic GBC patients [134]. Another study reported that *TFAP2A* overexpression plays a significant role in the regulation of malignant transformation and ferroptosis in GBC [133]. The identified TFs from the above-mentioned reports are mostly associated with metastatic invasion. Metastatic invasion is associated with epithelial–mesenchymal transition (EMT), which is one of the chief hallmarks of cancer progression that leads to metastatic spread and helps in promoting cancer cell plasticity, and contributes to both tumor initiation and metastasis. Further studies are required to identify potential TFs in GBC that can serve as diagnostic/prognostic/therapeutic biomarkers.

2.6 Role of systems biology in identifying pathological signatures in cancer

Systems biology is defined as the study of complex interactions in biological systems and the associated properties that result from these interactions. Systems biology in cancer aims to acquire a more comprehensive understanding of cancer formation and progression [135]. Advances in high-throughput, cost-effective, and tissue-sparing technologies that can examine tumors at various levels, paired with high-speed computer resources, have permitted a shift in the research paradigm toward an integrated systems biology approach [136]. These new technologies have substantially increased our capacity to create strong datasets and combine the data into a holistic perspective that is far more than the sum of the parts. Systems biology is a 'data-driven' discipline that requires enormous volumes of high-quality data from different sizes and ideas to develop robust and predictive models that can describe the complexity of the cell and its surroundings. Understanding drug resistance mechanisms, prediction of successful combination

therapy, and development of predictive biomarkers to boost the response rate to targeted treatments are all areas where systems biology approaches are widely used. The advancement of high-throughput techniques such as Next generation sequencing (NGS), RNAseq, and others have resulted in a dramatic shift in the studies of biological systems from a 'one gene model' (i.e., focusing on the identification of individual genes and proteins and pinpointing their roles in the cell) to a ‘multiple gene models' (i.e., the belief that molecules rarely act alone and biological entities are 'systems' - collections of interacting parts), which has resulted into development of many 'large-scale biology projects'. The implementation of large-scale biological initiatives is becoming more common as these technologies become more economical and accessible. Massive volumes of biological data have been generated since the advent of systems biology, and this trend is projected to continue in the future. High-throughput data is more comprehensive and unbiased than one-on-one biological data. This high-throughput research approach has significantly revolutionized the field of cancer research [137].

Cancer systems biology is the application of systems biology approaches to the analysis of how normal cells' intracellular networks are perturbed during carcinogenesis to develop effective predictive models that can aid scientists and clinicians in the validation of new therapies and drugs. High-throughput methods enable cellular and tissue-level genomic investigations of mutation rearrangements, copy number variations, and methylation, as well as rigorous analysis of RNA, and miRNA expression data, protein levels, and metabolite levels [138]. In recent years, extensive molecular profiling of tumors has greatly increased our understanding of the molecular pathophysiology underlying cancer development and progression. Individual tumors can be driven by substantially diverse molecular alterations, even if they share malignant markers and cells of origin. Tumors developing from the same organ or tissue have been revealed to have various molecular subtypes, which are frequently associated with different treatment responses and clinical outcomes [139]. As a result, traditional cancer therapy should be developed to accommodate more customized therapeutic techniques, in which treatment is assigned to each patient based on their individual molecular abnormalities. Precision and personalized medicine have this as their primary goal [139-140].

2.7 NGS-based transcriptomic studies for precision oncology

Due to remarkable progress in the field of genomics, an in-depth understanding of oncology has changed significantly in recent years [**Figure 2.4**]. With the completion of the Human Genome Project and the advancement of NGS methods, the structure and functions of the human genome as well as the pathways and processes regulating gene expression have been widely studied in the context of cancer genomics [141-142]. The increasing knowledge of the genetic basis of cancer development provides new insights into the molecular underpinnings of tumor development and targeted treatment [143-144]. The NGS has ushered in a new era of transcriptome profiling. It gives numerous possibilities to conduct simultaneous investigations of thousands of genes and analysis of complex molecular systems involved in cancer and thus offers a significant contribution to precision medicine [145]. NGS technology, initially launched in 2004, can be used in a variety of ways, including whole-genome and exome sequencing, RNAseq, which is now considered the "gold standard" of transcriptome analysis. RNAseq has been widely employed in gene expression studies in several cancers and is an important approach to explore molecular aberrations, identifying tumor biomarkers, and developing new therapeutic targets [145-146].

Transcriptomics has experienced rapid advancement in recent years [147,141]. A transcriptome is defined as a collection of all the RNA molecules transcribed from the genome in a specific cell at a specific developmental stage and under specific physiological or pathological conditions [148]. A transcriptome consists of protein-coding RNAs (pcRNAs), also known as messenger RNAs (mRNAs), and ncRNAs, each of which performs a unique set of tasks in the cell and responds differently to external stimuli [149]. The transcriptome profile can be seen as a snapshot of a transient cell state, and therefore, the analysis will offer insights into gene function, genome plasticity, gene expression regulation, and individual transcript change [150-152]. Therefore, transcriptome analysis is regarded as a promising tool for investigating continually changing cancer cells at the molecular level. The findings from the transcriptome study have directly contributed to the extensive development of precision oncology [153]. Numerous transcriptomics-based studies have been conducted to date to profile the gene expression and identify therapeutic/diagnostic targets in breast cancer through molecular categorization [154-158]. Perou et al., (2000) performed one of the first successful attempts at gene expression-based

characterization. Individual gene expression patterns were identified in 65 breast cancer samples acquired from 42 patients [159]. Four molecular clusters of breast cancer were identified, each with a comparable expression signature: ER+/luminal-like, basal-like, Erb-B2+ (Her-2/neu), and normal-like breast cancers. The clusters identified in this investigation correspond closely to breast tumor immunohistochemical markers: estrogen receptors, Her-2, and Ki-67, and should thus be addressed as different disorders [160-161].

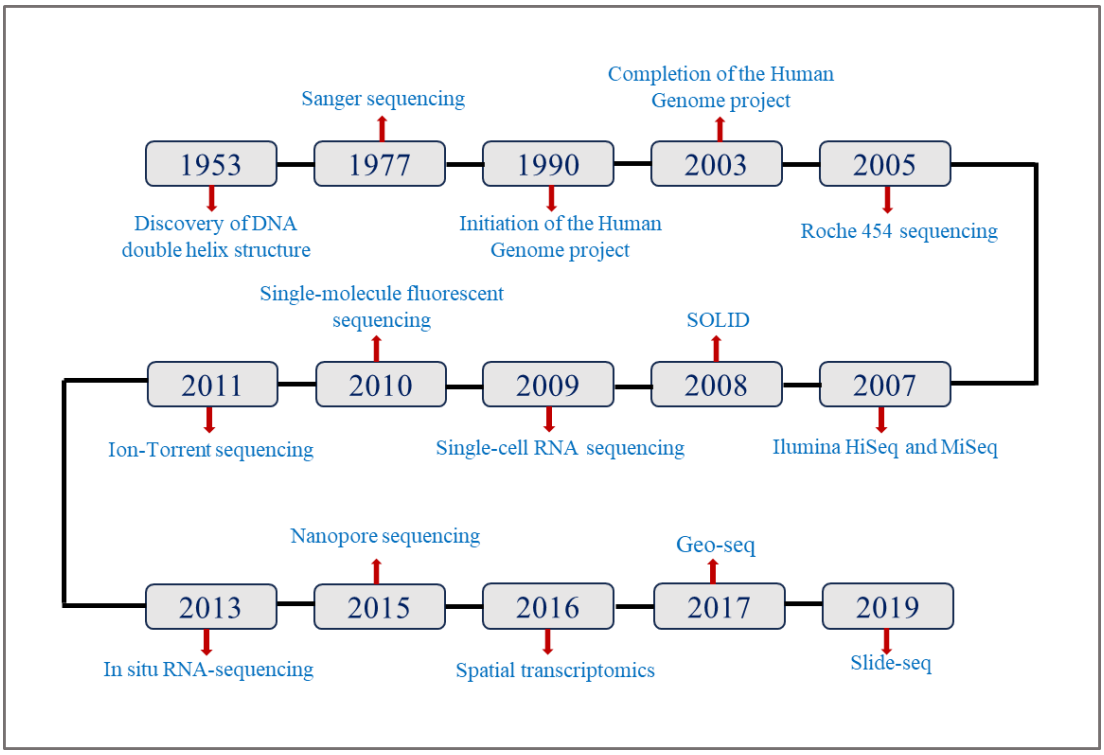

Figure 2.4: The roadmap of development of RNA sequencing technologies (Reproduced from [180])

2.8 Transcriptomic-based studies in GBC

Efforts have been made to understand the molecular players involved in imparting GBC pathogenicity. Multiple gene expression studies [162-177] have investigated the differential expression profiles between the normal gallbladder and GBC [**Table 2.3**]. However, the precise molecular mechanism(s) behind GBC development from GSD and de novo GBC pathogenesis is still unknown. So far, no biomarker has been identified for therapeutic selection in the treatment of GBC. A lack of GBC-specific markers, as well as the absence of targeted therapy, contribute

to late diagnosis and poor prognosis, which frequently leads to poor clinical outcomes [178]. Furthermore, most of the available literature has focused on the genetic landscape of GBC in terms of susceptibility [179]. Thus, a thorough understanding of the expression profile of GBC may aid in the identification of potential biomarkers, resulting in better disease management and therapy selection.

Table 2.3: List of OMICs scale GBC datasets available at NCBI and ENA databases.

	Accession ID	Study	Study group	Ref
High-throughput sequencing (RNA sequencing)	SRP227857/ GSE139682	Study on the gene expression profile of GBC.	China	[162]
	SRP226150	Study on transcriptome profile of GBC and GSD with three follow-up periods.	China	[163]
	SRP200495/ GSE132223	Study on the gene expression profile of GBC liver metastasis.	China	[164]
	SRP306808/ GSE166915	Study on whole transcriptomes exosomal signatures on GBC and xantho-granulomatous cholecystitis.	China	[165]
	SRP374181	Multistage transcriptome profile of GBC and associated chronic inflammation.	China	[166]
	SRP110184/ GSE100363	Study on differentially expressed circRNA in GBC and matched normal samples.	USA	[167]
	DRP008090	Study on characterization of GBC tumor microenvironment.	Japan	[168]
	SRP372089	Single-cell atlas of diverse immune populations in the advanced biliary tract cancer microenvironment.	China	[169]
	GSE90001	Study on the microRNA expression profile of GBC.	China	[170]
Microarray	GSE76633	Study on the lncRNA expression profile of GBC.	China	[171]
	GSE106671	Gene expression signatures of highly metastatic GBC cells.	China	[173]
	ERP108145	FGF19-FGFR4 promotes the progression of gallbladder carcinoma in a biliary autocrine pathway dependent on the GPBAR1-EGR1 axis.	China	[172]

Genome and exome sequencing	ERP108145	Whole exome sequencing of human tumors and cell lines to investigate how ERBB2 and KRAS Alterations mediate response to EGFR inhibitors in early-stage GBC.	India	[174]
	SRP265406	The whole exome mutational landscape of GBC.	China	[175]
	ERP015935	An Indian germline variant dataset derived from a whole exome sequence.	India	[176]
	SRP272844	Genomic characterization biliary tract intraepithelial neoplasia and GBC.	China	[177]

2.9 Gaps in Research

GBC is a highly aggressive and lethal form of cancer, ranking as the most common biliary tract cancer globally. Characterized by a complex etiology, poor prognosis, and delayed diagnosis, GBC poses significant challenges in understanding its pathophysiology, particularly in GS-independent GBC cases. The prevalence of GBC is notably high in the northern and NE regions of India, with a higher incidence rates among females in the NE parts. Despite being one of the most common cancers in this region, the pathophysiology of GBC, especially GS-independant GBC, remains poorly understood, leading to a lack of effective therapeutic options with low response rates.

Cholecystectomy after incidental gallstone diagnosis stands as the only preventive measure currently available. The absence of specific biomarkers for GBC further complicates diagnosis and therapy, as the understanding of genetic aberrations and associated pathways remains limited. Despite ongoing research in hepatobiliary cancer, GBC-specific candidate markers for diagnosis and therapy are inadequately defined. Additionally, the scarcity of OMICs-scale datasets, particularly from high-risk GBC populations, hinders comprehensive insights. In the era of high-throughput sequencing, exploring the transcriptomic landscape emerges as a crucial avenue. Such characterization holds the potential to identify important molecular signatures, aiding in the early identification of high-risk individuals, facilitating prompt diagnosis, predicting prognosis, and identifying therapeutic targets for GBC patients. This thesis is an attempt to address the existing gaps in our understanding of gallbladder carcinoma by employing an integrative systems biology approach.

The current state of knowledge about genetic and molecular alterations in GBC is extremely limited, particularly in India, where the incidence and mortality rates of GBC patients are extremely high. We hypothesize that some specific molecular signatures and pathways govern GBC with GS and GBC without GS pathogenicity independently. Based on the gap in research, the thesis aims to identify potential molecular players in GBC+GS and GBC group that drive gallbladder carcinogenesis.

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