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Genomic landscape of gallbladder cancer: insights from whole exome sequencing

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Background: Gallbladder cancer (GBC) is a common gastrointestinal malignancy noted for its aggressive characteristics and poor prognosis, which is mostly caused by delayed detection. However, the scarcity of information regarding somatic mutations in Indian patients with GBC has hampered the development of efficient therapeutic options. In the present study, the authors attempted to bridge this gap by revealing the mutational profile of GBC.

Materials and methods: To evaluate the somatic mutation profile, whole exome sequencing (WES) was performed on 66 tumor and matched blood samples from individuals with GBC. Somatic variant calling was performed using GATK pipeline. Variants were annotated at pathogenic and oncogenic levels, using ANNOVAR, VEP tools and the OncoKB database. Mutational signature analysis, oncogenic pathway analysis and cancer driver genes identification were performed at the functional level by using the maftools package.

Results: Our findings focused on the eight most altered genes with pathogenic and oncogenic mutations: TP53, SMAD4, ERBB3, KRAS, ARID1A, PIK3CA, RB1, and AXIN1. Genes with pathogenic single nucleotide variations (SNVs) were enriched in oncogenic signaling pathways, particularly RTK-RAS, WNT, and TP53 pathways. Furthermore, our research related certain mutational signatures, such as cosmic 1, cosmic 6, and cosmic 18, 29, to known characteristics including patient age and tobacco smoking, providing important insights into disease etiology.

Conclusions: Given the scarcity of exome-based sequencing studies focusing on the Indian population, this study represents a significant step forward in providing a framework for additional in-depth mutational analysis. Genes with substantial oncogenic and pathogenic mutations are promising candidates for developing targeted mutation panels, particularly for GBC detection.

Keywords: gallbladder cancer (GBC), genome analysis toolkit (GATK), OncoKB, whole exome sequencing (WES)

Introduction

Gallbladder Cancer (GBC) is the most common biliary tract cancer[1] (BTC), and is distinguished by its aggressive nature and nonspecific symptoms, with the majority of cases being found inadvertently at advanced clinical stages[2]. According to the GLOBOCON 2023 report, GBC ranks 22nd globally among all malignancies, with a reported incidence of 12,2491 cases. Heterogeneity is the primary reason for variance in the prevalence of GBC across geographical regions, with particularly high rates in South America and Asian countries[3,4]. In India, GBC is the most prevalent Gastrointestinal Tract cancers[5], ranking 19th among all cancers, with 21,780 newly reported cases and 16,407 deaths. GBC incidence exhibits regional disparities within India, being higher in northeastern regions, and sex-wise occurrence is more susceptible in females than in males[6]. Owing to the multifactorial etiology of GBC, factors such as sex, age, chronic inflammation, gallbladder polyps, and lifestyle habits contributes significant level[7]. Gallstones are thought to be the main risk factor, although according to new research, only 1–3% of gallstone patients develop GBC. Furthermore, 10–15% of individuals have never had gallstones[8,9]. Numerous exome-based sequencing investigations have yielded significant information regarding the genetic makeup of GBC across a range of groups. The first Caucasian GBC exome study[10], found a significant prevalence of TP53 (62.5%, 5/8) in the GBC cases analyzed. Subsequent another research[11], comprising WES and targeted

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deep sequencing of GBC samples from a Chinese population, identified TP53 as the most frequently mutated gene (47.1%), followed by KRAS (7.8%), ERBB3 (11.8%), and ErbB signaling dysregulation at a significant level. A BTC spectra study identified ERBB and RB cell cycle as the prevalent pathways, along with TP53 mutations in GBC cases[12]. A follow-up exome study on GBC identified frequent ERBB2/3(9.8%,11.8%) mutation[11,13]. Further WES studies across different ethnicities corroborated these findings, with TP53 mutations recurring prominently in GBC, as highlighted in Japanese[14], US[15], and Chinese cohorts[16,17] (29.3%, 27/92; 59%, 22 samples, respectively). Novel insights into GBC pathogenesis were obtained, including the significant role of ELF3 mutations in patients from diverse geographical regions, incorporated 64 patients of Indian origin[18]. Notably, among studies focusing on the Indian population[19], somatic alterations implicating ERBB2 and KRAS genes have been unveiled. Another study[20], performed WES ($n=11$), and found that TP53(6/10), ERBB2, and OBSCN (3/10) were the major mutated genes. One study[21], analyzed the transcriptomic and genomic profiles ($n=38$) of patients with GBC and identified TP53(47%), ELF3(13%), and ARID1A (11%) as the major mutated genes along with alterations in TGF-β pathway. A comprehensive study[22] ($n=164$) of GBC cases, found TP53 to be the most frequently mutated gene, with alterations in p53 and cell cycle pathways. Another study[23] examined patients with GBC ($n=7$) and TP53 mutations were found to be prevalent, along with alterations in KM2TC, CDKN2A, and ARID1A in a subset of patients. Furthermore, a WES study[24] on 11 patients with GBC, revealed CTNNB1 and ARID2 mutations in a significant proportion of cases, along with concurrent TP53 and ERBB3 alterations in a subset. Another exome-based study[25], identified SYNE1 and TP53 as highly mutated driver genes in a GBC study.

Advancements in genomics have demonstrated the ability to deal with tailored therapies, which includes the use of mRNA vaccines and nanovaccines. mRNA vaccines in cancer attempt to trigger the immune system to recognize and target cancer cells, and nanovaccines provide a promising path for cancer therapy by improving vaccine distribution and efficacy. Their capacity to target specific antigens, protect and transport them efficiently, and strengthen the immune system makes them an effective tool in the battle against cancer[26,27]. Furthermore, advanced AI developed by OpenAI has the potential to play a key role in cancer research by being integrated into decision support systems, allowing researchers to quickly access evidence-based guidelines, treatment protocols, and diagnostic criteria[28]. Identification of biomarkers and broader research in these fields, may be applied to GBC and helps in the improvement of treatment outcomes. This study identified mutations linked to oncogenic and pathogenic genes along with mutational signatures. Moreover, this study provides insights into oncogenic pathways, offering valuable guidance for potential targeted therapeutic approaches in Indian patients with GBC.

HIGHLIGHTS

- Provides a comprehensive analysis of the somatic mutational landscape of gallbladder cancer (GBC) using whole exome sequencing.
- Findings spotlighted the eight most altered genes bearing pathogenic and oncogenic relevant mutations.
- This study identified mutational signatures linked to age and tobacco addiction-that are prevalent among patients diagnosed at later stages.
- Results underscore the intricate and heterogeneous nature of GBC within our cohort.
- The discovered mutations have high potential for inclusion in the GBC diagnostic gene panels.

Materials and methods

Study population

This study was approved by the ethical committees of the host institute. All samples were collected after obtaining written informed consent from patients. Fresh-frozen tumor tissues and matched peripheral blood (PB) samples were obtained during surgery and stored at – 80°C for further processing as described previously[29]. A total of 66 paired samples were collected and histologically confirmed. Of these, 24 were male and 42 were female, with a median age of 53 years. According to the AJCC 8th edition, 17, 22, 22, and 5 patients had Stage I, II, III and IV disease, respectively. Patient data, primarily from those diagnosed with GBC were reviewed, and the associated clinical data are summarized in Table 1.

DNA extraction, library preparation, and sequencing

Genomic DNA (gDNA) from the stored tumor tissue and matched blood was extracted using a Qiagen kit, in accordance with the manufacturer's protocol. To ensure the integrity of gDNA, quantity and quality assessments were performed using a Qubit fluorimeter and agarose gel electrophoresis, respectively. Upon preliminary analysis, 200 ng of gDNA was used for library preparation using the Agilent SureSelectXT Target Enrichment System, according to the manufacturer's instructions. Finally, an enriched indexed library was captured and assessed using a Bioanalyzer assay, followed by 150 bp paired-end sequencing using the Illumina NovaSeq 6000 system.

Preprocessing of raw reads

Quality assessment of raw data reads was conducted using FASTQC[30], followed by adapter trimming using Trimmomatic[31]. High-quality trimmed reads were mapped to the UCSC human genome (GRCh38.p12) using Burrow-Wheeler Aligner BWA-MEM[32]. Further alignment processing, including quality control (QC) metrics, duplicate marking, and base quality score recalibration, was performed using the Genome Analysis Toolkit[33] (GATK).

Panel of normals, somatic variant calling

Panel of Normals (PONs) is the collection of all germline samples in the vcf file format. PONs were created using Mutect, on individual normal samples and combining all normal variant calls including the criterion that specific germline variant sites should be present in at least two normal samples. GetPileupSummaries and Calculate Contamination tools of GATK were used for cross-sample contamination analysis, and identified somatic variants were filtered using FilterMutectCalls.

Table 1

Clinical characteristics of 66 GBC samples.

Sample_ID	Sex	Age	Gallstone	Jaundice	Addiction	Tumor stage
GBC_1	Female	49	Yes	No	No	Stage I
GBC_2	Female	68	No	No	No	Stage IIB
GBC_3	Male	31	Yes	No	Tobacco chewing	Stage IIA
GBC_4	Female	59	No	Yes	No	Stage IVB
GBC_5	Female	56	No	No	No	Stage IIA
GBC_6	Male	63	No	No	No	Stage IIIB
GBC_7	Female	64	No	No	No	Stage IIB
GBC_8	Male	65	No	Yes	No	Stage IIIB
GBC_9	Female	56	No	No	No	Stage I
GBC_10	Female	56	No	No	Tobacco chewing	Stage IIB
GBC_11	Female	40	Yes	Yes	No	Stage IIIB
GBC_12	Male	43	No	No	Smoking, Tobacco chewing	Stage IIA
GBC_13	Male	59	No	Yes	Tobacco chewing, alcohol	Stage IIIB
GBC_14	Female	34	No	Yes	No	Stage IIIA
GBC_15	Female	50	Yes	No	Smoking	Stage I
GBC_16	Female	71	No	No	No	Stage IIIB
GBC_17	Male	56	No	No	Smoking, Tobacco chewing, alcohol	Stage IIIB
GBC_18	Male	30	No	Yes	Tobacco chewing, alcohol	Stage IVB
GBC_19	Male	44	Yes	Yes	Alcohol	Stage IVA
GBC_20	Male	63	Yes	No	No	Stage IIIA
GBC_21	Female	48	No	No	Smoking	Stage IIIA
GBC_23	Female	55	No	No	No	Stage IIIB
GBC_24	Female	52	No	No	No	Stage IIIB
GBC_25	Female	45	No	No	No	Stage I
GBC_26	Male	34	Yes	Yes	Tobacco chewing	Stage IIIA
GBC_27	Male	68	No	No	Tobacco chewing	Stage IIB
GBC_28	Male	71	Yes	No	No	Stage IIIB
GBC_29	Female	57	No	No	Smoking	Stage IVB
GBC_30	Female	40	Yes	Yes	Smoking	Stage I
GBC_31	Female	72	Yes	Yes	Smoking	Stage IVB
GBC_32	Female	54	Yes	Yes	No	Stage I
GBC_33	Male	77	No	No	No	Stage IIIA
GBC_34	Male	50	No	No	Tobacco chewing	Stage IIA
GBC_35	Female	63	No	No	No	Stage I
GBC_36	Female	50	No	Yes	No	Stage IIIA
GBC_86	Female	39	No	Yes	No	Stage III
GBC_87	Female	35	No	No	No	Stage IIB
GBC_103	Female	43	No	No	Smoking, Tobacco chewing	Stage IIA
GBC_104	Male	27	No	No	Tobacco chewing, alcohol	Stage I
GBC_113	Male	27	No	No	No	Stage I
GBC_114	Female	51	Yes	No	Smoking	Stage IIA
GBC_132	Male	57	Yes	No	No	Stage I
GBC_133	Female	48	Yes	No	Smoking	Stage IIIB
GBC_134	Female	27	No	No	No	Stage IIB
GBC_135	Female	49	Yes	No	No	Stage I
GBC_136	Male	65	No	No	No	Stage IIA
GBC_137	Female	74	No	No	N o	Stage IIB
GBC_138	Male	64	No	No	No	Stage IIIA
GBC_139	Female	40	Yes	No	No	Stage IIIB
GBC_140	Female	45	Yes	No	No	Stage IIIB
GBC_141	Female	39	Yes	No	No	Stage IIIB
GBC_142	Male	35	No	No	No	Stage IIA
GBC_144	Female	25	No	No	No	Stage I
GBC_145	Female	51	Yes	No	Smoking	Stage IIA
GBC_146	Female	32	No	No	No	Stage I
GBC_147	Female	65	Yes	No	No	Stage I
GBC_148	Female	68	Yes	No	No	Stage IIB
GBC_149	Male	45	Yes	No	No	Stage IIIB
GBC_150	Female	40	Yes	No	No	Stage I
GBC_151	Female	64	No	No	Smoking	Stage I
GBC_152	Female	80	Yes	No	No	Stage IIA
GBC_153	Male	34	No	No	No	Stage I

Table 1

(*Continued*)

Sample_ID	Sex	Age	Gallstone	Jaundice	Addiction	Tumor stage
GBC_154	Female	42	Yes	No	Tobacco chewing	Stage IIB
GBC_155	Female	50	Yes	Yes	Smoking	Stage IIB
GBC_156	Male	61	Yes	No	No	Stage IIIA
GBC_157	Male	64	Yes	No	No	Stage IIA

Somatic variants annotation with clinical and oncogenic relevance

We implemented SnpEff[34], Annotate variation[35] (ANNOVAR), and Variant Effect Predictor[36] (VEP) for annotation and prioritization of variants based on their clinical significance. Initially, all variant files were annotated with dbSNP155[37] and 1000 genomes[38] databases using the SnpSift function of SnpEff. Variants with Minor allele frequency (MAF ≤ 0.01) and South Asian Allele frequency (SAS_AF ≤ 0.01) were selectively considered. Using ANNOVAR, mutations were prioritized and filtered through four deleterious predictors with associated scores: Sorting Intolerant from Tolerant[39] (SIFT <0.05 as cutoff), Functional Analysis through Hidden Markov Model[40] (FATHMM; D-deleterious, T-tolerated), Polymorphism Phenotyping v2[41] (PolyPhen-2; Polyphen-2 < 0.9 as probably damaging(D)), and likelihood ratio test[42] (LRT; D-Deleterious, N-neutral, U-Unknown). Disease-specific variants were annotated using ClinVar[43] (2022123139) and Catalog of Somatic Mutations In Cancer[44,45] (COSMIC_95) databases were used for mapping with known somatic mutations. Clinical interpretation of the variants, such as - benign, variants of uncertain significance (VUS), Pathogenic, and Likely Pathogenic, was performed according to the American College of Medical Genetics (ACMG) and the Association for Molecular Pathology (AMP) guidelines, utilizing the InterVar database[46].

Somatic mutations were meticulously annotated in our study based on diagnostic, therapeutic, and prognostic evidence levels, using the OncoKB database[47]. A cutoff of SIFT (< 0.05) and Polyphen2 (< 0.9) scores were applied to identify the deleterious effect of oncogenic mutations. Subsequently, we selectively considered variants annotated from the somatic mutation cancer database, which demonstrated a pathogenic or likely pathogenic effect with oncogenic activity. In addition to in-house shell scripts and R scripts, Maftools[48] was used for summarizing, visualizing, and analyzing the maf files.

Cancer driver genes identification

MutSigCV[49] was used to identify the genes associated with cancer driver activity in 66 GBC samples. Considering the approach used for identifying significant genes, the background mutation rate (BMR) was estimated using the observed count, coverage count of mutations per gene, and mutation category, using the pre-processed mutation data, coverage data, and covariate data. Genes that harbored a significant P (< 0.01) and q value threshold of <0.1 were considered as significant cancer driver genes.

Somatic mutational signature analysis, tumor mutational burden analysis

Cancer progression leads to a characteristic mutational pattern that can reveal underlying mutagenic processes[50,51]. Implementing 'de novo' approach, involving frequency matrix generation and non-negative matrix factorization (NMF) decomposition to extract signatures and compared to reference signatures from COSMIC based on cosine similarity. Additionally, APOBEC enrichment was performed for individual tumor samples as described in a study[52]. We applied the 'tcgaCompare' function from the maftools package for tumor mutation burden (TMB) analysis and compare it to the 33 TCGA cancer types.

Oncogenic pathway analysis

Mutated genes were assigned to oncogenic signaling pathways based on data from previous TCGA based study[53]. We evaluated 10 canonical signaling pathways with frequent genetic alterations, by using the 'Oncogenic Pathways' module of maftools. Additionally, Pathwaymapper[54] and the pathway template from TCGA PanCanAtlas[53] were used to generate mutated signaling pathways.

Results

Mutational profile of GBC patients

A total of 22,285 variants and 16,834 protein-altering-based somatic mutations were identified in our cohort. C > A substitutions were dominant (more than 50% of samples), followed by C > T substitutions (40% of samples) (Figure S1A, B; Supplemental Digital Content 1, http://links.lww.com/JS9/D293). The exonic region carried the highest number of mutations ($n = 21,844$) (Fig. 1A, B). In our study, nonsense and silent mutations were the next most common variant type, after missense mutations (Table S1, Supplemental Digital Content 2, http://links.lww.com/JS9/D294). TP53 (47%), MUC16 (30%), SYNE1 (29%), CTNNB1 (27%), SMAD4 (26%), and OBSCN (24%) were the most frequently mutated genes in the study cohort (Figure S2; Supplemental Digital Content 1, http://links.lww.com/JS9/D293).

Cancer driver genes in GBC

We identified 11 genes with notable significance scores based on both p and q values (Table S2, Supplemental Digital Content 3, http://links.lww.com/JS9/D295). TP53 exhibited the highest mutation frequency (45%), followed by ELF3 (17%), CTNNB1 (27%), AXIN1 (11%), ERBB2 (21%), SMAD4 (24%), ARID1A (15%), RB1 (11%), ARID2 (14%), PTEN (8%), and ERBB2 (Fig. 2).

Genes associated with pathogenic variants

A total of 17,976 variants were categorized according to the ACMG and AMP guidelines. Among them, 12,499 were VUS

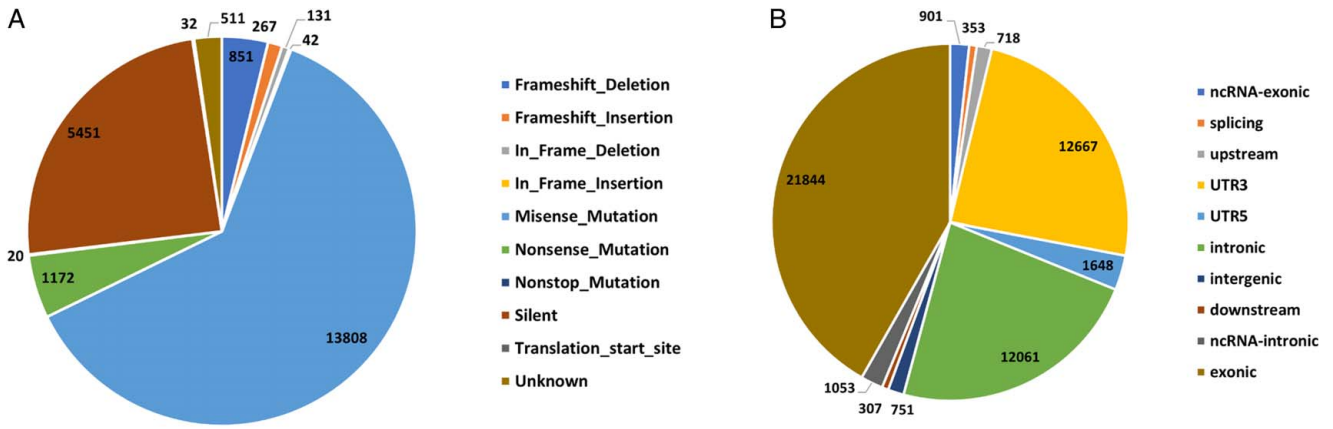

Figure 1. Distribution of Somatic variants in 66 GBC Samples. (A) Types of mutations identified in GBC cohort. Highest occurence of Missense mutation is observed. (B) Classification of overall variants observed in GBC samples. Exonic variants are highest in number followed by the UTR3 regions and intronic regions.

variants and 4996 were likely benign variants. Additionally, 301 variants were pathogenic, and 180 were likely pathogenic (Figure S3A, B; Supplemental Digital Content 1, http://links.lww.com/JS9/D293). Missense, nonsense, stop-gain, and start-loss mutations (481 mutations) were predominant in 61 GBC samples. These pathogenic variants were distributed across 393 genes,

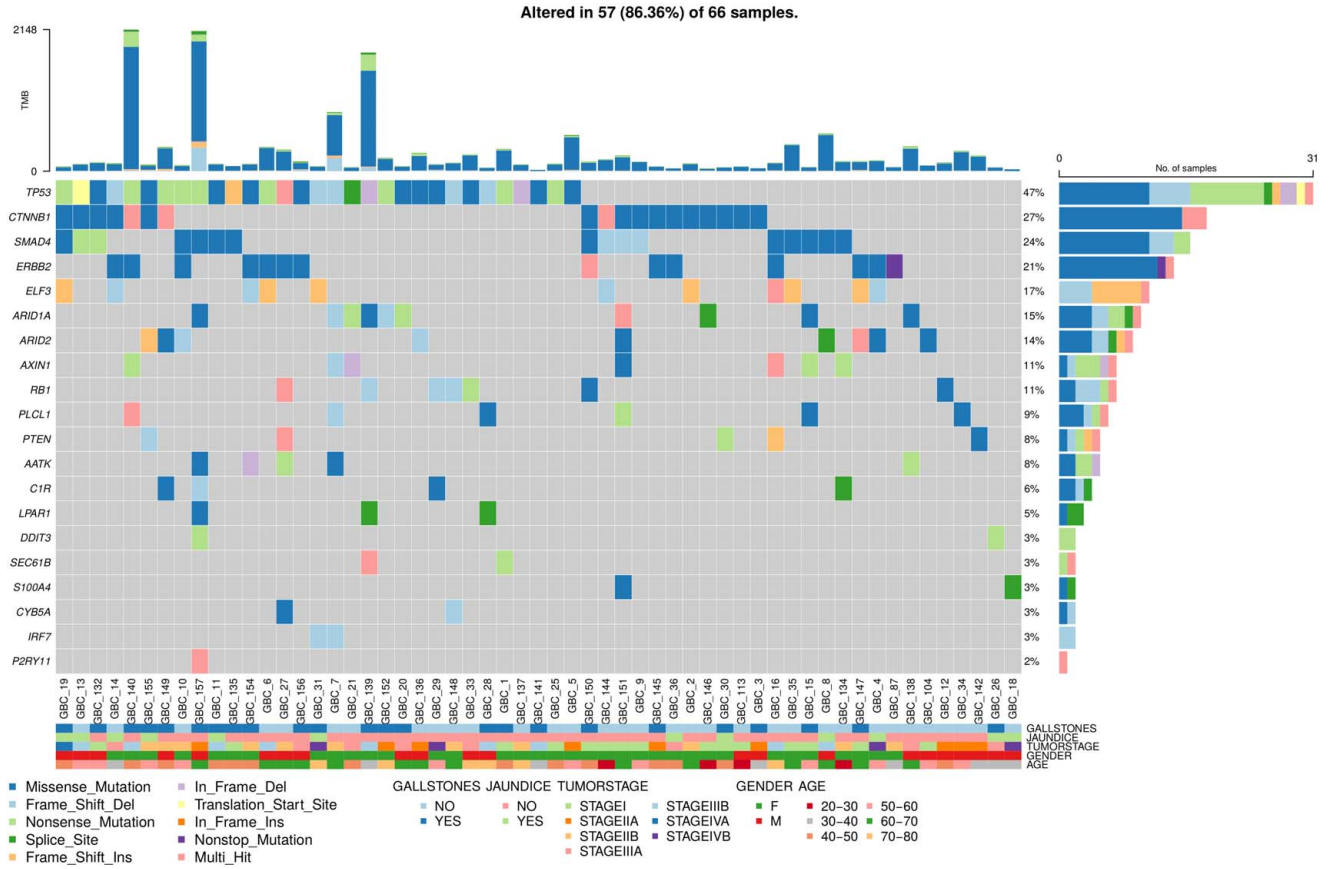

Figure 2. Oncoplot illustrating the top genes with potential driver variants, correlated with sample, age, sex, tumor stage, gallstone presence, and jaundice status in 57 GBC samples. Each column represents a distinct GBC sample, while each row denotes a specific gene. Colored squares indicate altered genes, whereas grey squares signify non-mutated genes. Variants are color-coded according to their mutation types. Genes marked as "Multi_Hit" denote those with multiple mutations within the same sample. The barplot at the top shows the tumor mutation burden (TMB), with colors representing different mutation types.

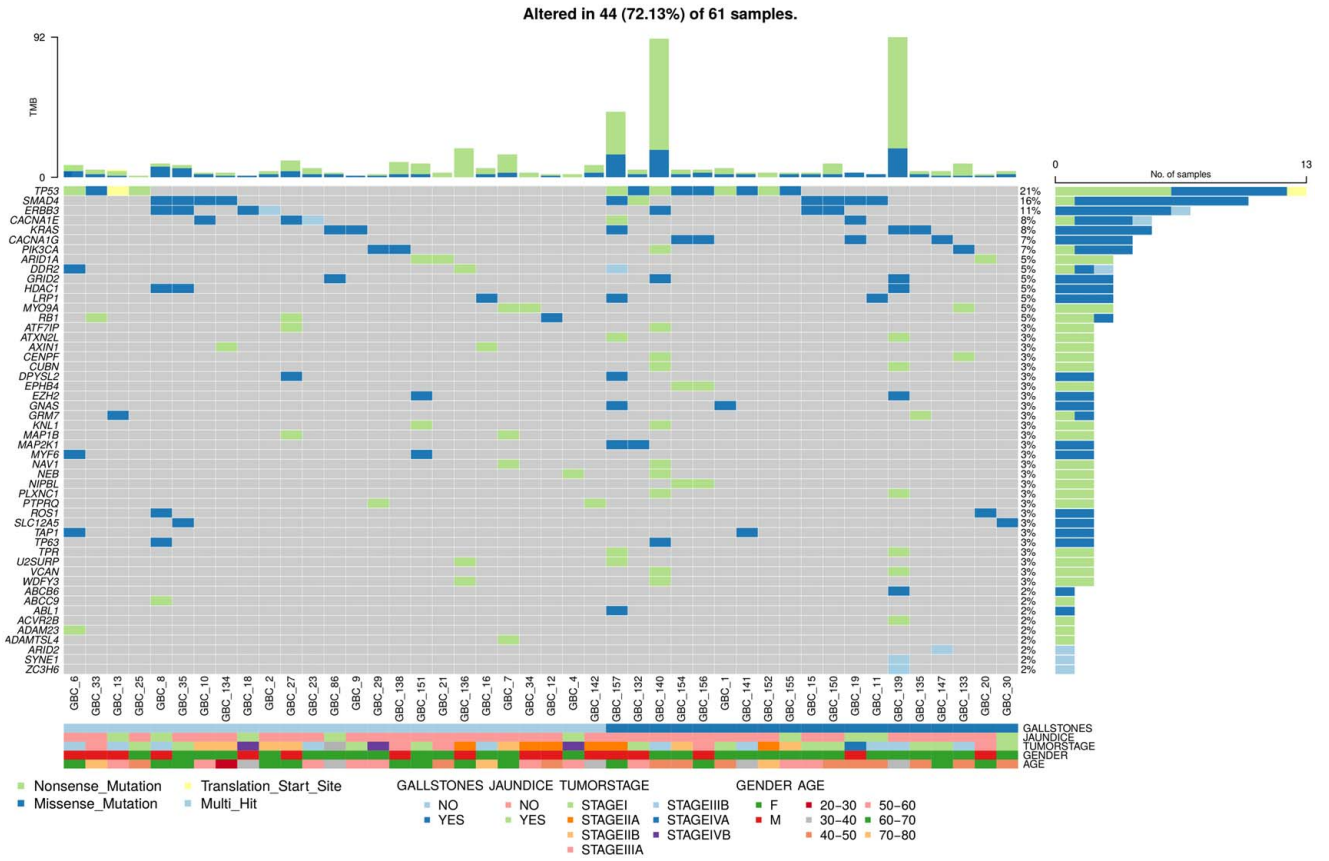

Figure 3. Oncoplot showing pathogenic gene variants and their distribution among 44 out of 61 gallbladder cancer (GBC) samples. These genes were selected based on ACMG classification, highlighting pathogenic and likely pathogenic variants. The gene variants are correlated with clinical features, including age, sex, tumor stage, gallstone, and jaundice status. Each column represents a GBC sample, and each row corresponds to a specific gene. Colored squares indicate mutated genes, while grey squares represent non-mutated genes. Different mutation types are distinguished by various colors. Genes marked as "Multi_Hit" contain more than one mutation in the same sample. The barplot at the top displays tumor mutation burden (TMB), color-coded by mutation type, while the barplot on the right shows the number of patients with mutations in each gene.

including TP53 (21%), SMAD4 (16%), ERBB3 (11%), CACNA1E (8%), KRAS (8%), and PIK3CA (8%) (Fig. 3).

In addition to identifying mutations, these findings were annotated using the OncoKB database. Of these 481 variants, 433 were effectively annotated, revealing associations with 349 distinct genes. Among these, 39 genes were definitively linked to oncogenic activity, accounting for 43 of the initially 61 samples. Intriguingly, 12 samples exhibited deleterious scores, although the OncoKB classified the variation in activity as uncertain. Moreover, six samples failed to exhibit significant scores, and their oncogenic activity remained undetermined. Of the 39 genes associated with oncogenic activity, only eight genes-TP53(21%), SMAD4(16%), ERBB3(11%), KRAS (8%), ARID1A (5%), PIK3CA (7%), RB1(5%), and AXIN1(3%), were found to be mutated in at least two samples (Fig. 4). Further annotation of these oncogenic mutations revealed varying therapeutic implications, diagnostic relevance, and prognostic significance (Table S4; Supplemental Digital Content 4, http://links.lww.com/JS9/D296).

TP53 was the most frequently mutated gene in our study, with both pathogenic and likely pathogenic mutations. TP53 mutations were found in 13 samples, including 6 male and 7 female individuals, most aged over 50 years, and had been diagnosed with Stage II or III GBC. Interestingly, only half of the patients had cholelithiasis, with six variants as pathogenic and seven as likely pathogenic activity. All TP53 missense and nonsense mutations were prevalent at prognostically significant levels (Px1, Px3). The TP53 mutations found in GBC cohort included p.K132T (Level_Px1, Px3), p.M133T (Level_Px1), p.H179R (Level_Px1), p.E285K (Level_Px1), p.G245S, and p.S127F (Level_Px1) (Table 2). SMAD4 was the second most frequently mutated gene, with pathogenic mutations found in nine samples. Among them, six were female and three were female, with a median age of 50 years. Most patients presented with Stage II and III GBC, and five patients with SMAD4 mutations also had cholelithiasis. Mutations such as p.D537Y, p.D493N, p.E330K, p.W509R, p.R361H, and p.G352V were discovered to be related with SMAD4 in the GBC cohort (Table S5A-E; Supplemental Digital Content 2, http://links.lww.com/JS9/D294).

ERBB3 mutations were prevalent in seven samples, affecting five female and two male patients. The median age of these patients was 50 years, and they predominantly belonged to Stages III and IV. ERBB3 oncogenic mutations include p.G284R, p.V104L, p.P262H, and p.V104M. Additionally, p.E298G was also reported.

KRAS mutations were detected in four samples: three female and one male patient. Two of these patients had cholelithiasis,

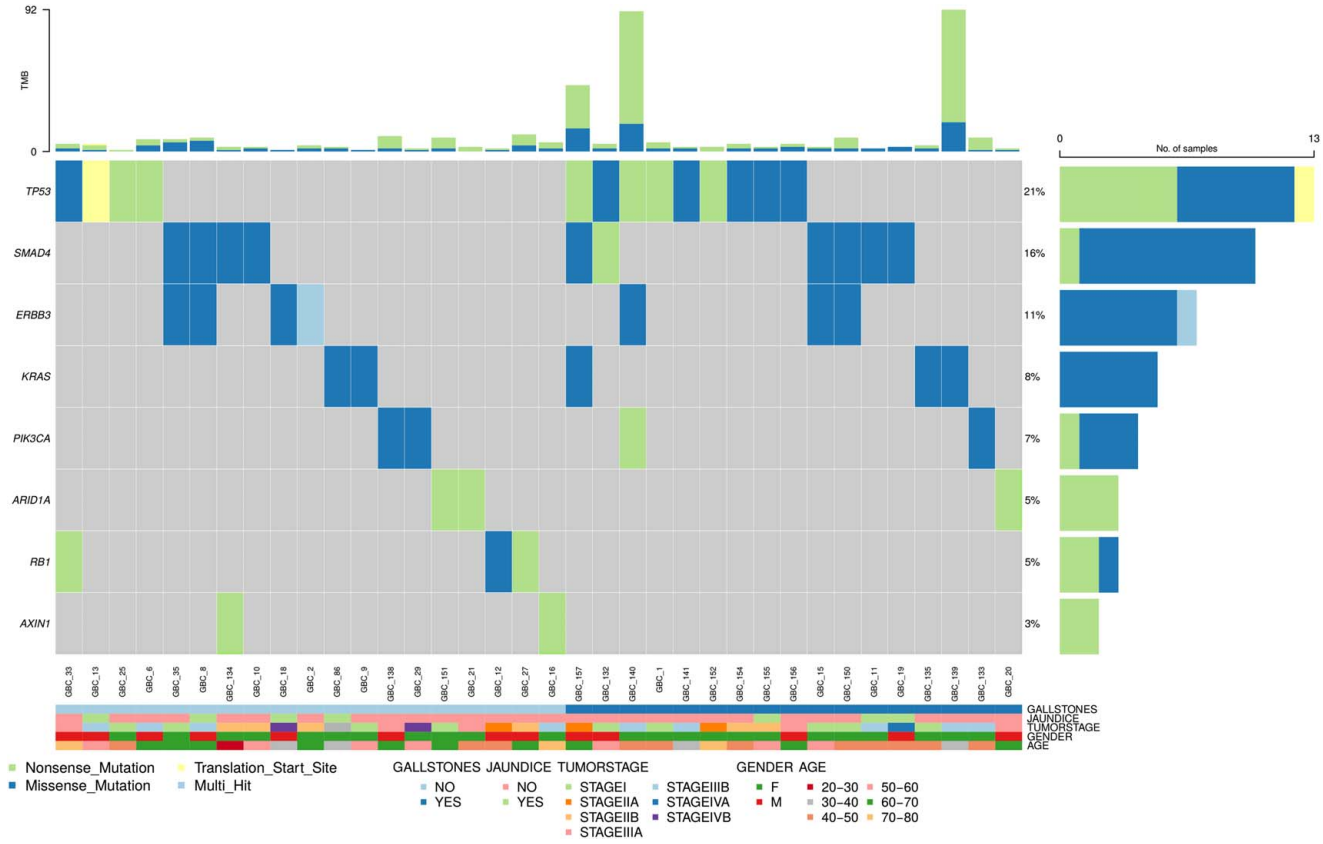

Figure 4. Oncoplot depicting the distribution of eight prevalent genes—TP53, SMAD4, ERBB3, KRAS, PIK3CA, ARID1A, RB1, and AXIN1—within the GBC cohort. These genes, mutated in at least two GBC samples, are noted for their oncogenic mutations which have implications for therapy, diagnosis, and prognosis. Gene variants are correlated with sample, age, sex, tumor stage, and status of gallstones and jaundice. Each column represents a distinct GBC sample, and each row corresponds to a specific gene. Colored squares indicate altered genes, while grey squares denote non-mutated genes.

Table 2

TP53 oncogenic mutations in GBC samples.

Gene	Chr position	Variant	Oncogenic	Variant in OncoKB; level of evidence	Description
TP53 (chr17)	7675217	p.K132T	Likely Oncogenic	Yes;	Identified in glioblastoma[58]
	7675214	p.M133T	Likely Oncogenic	Level-Px1[a]	Unregulated inflammatory response studied in breast cancer[59]
	7674894	p.R213*	Likely Oncogenic	Yes; Level-Px1	Truncating mutation and promote cancer cell proliferation[62]
	7673767	p.E285K	Likely Oncogenic	No;	Expression in yeast and human cancer cells is associated with loss of
	7673537	p.Q331*	Likely Oncogenic	Level-Px1	function[61] Truncating mutation and its alterations are predicted to be
	7674230	p.G245S	Oncogenic	Yes; Level-Px1	inactivating and are associated with poor prognosis.
	7675232	p.S127F	Likely Oncogenic	No;	This mutation in cell lines that lack TP53 expression did not enhance the
	7675119	p.Q165*	Likely Oncogenic	Level-Px1	TP53-mediated transcriptional activity[62]
	7673740	p.E294*	Likely Oncogenic	Yes;	Mutation is identified in xeroderma pigmentosum and is statically
	7676097	p.W91*	Likely Oncogenic	Level_Px1	significant hotspot[63]
	7675076	p.H179R	Likely Oncogenic	Yes;	Truncating mutation and its alterations are predicted to be inactivating and
				Level_Px1	are associated with poor prognosis[63]
				No; Level_Px1	Truncating mutation and its alterations are predicted to be inactivating and
				No; Level_Px1	are associated with poor prognosis[63]
				No;	Expression of this mutation failed to induce expression of genes involved in
				Level_Px1	apoptosis and cell cycle arrest[60]
				Yes;	
				Level_Px1	

[a]FDA and /or professional guideline-recognized biomarker prognostic in this indication based on a well powered study/studies.

and their GBC stages ranged from I to III. KRAS has demonstrated a spectrum of therapeutic implications ranging from levels 2 to 4, along with resistance (level R1) and diagnostic relevance (level Dx2). The mutations identified were p.G12V, p.V14I, and p.G12D.

PIK3CA mutations were found in three samples, including two female and one male patient, all categorized as Stage III/IV GBC cases, with a median age of 40 years. PIK3CA mutations have been linked to Level 1 therapeutic implications. The p.R38H mutations, p.E545K, and p.E365K has been associated with PIK3CA.

ARID1A mutations were detected in three samples, two females and one male patient, two of whom had Stage I GBC and the remaining one had Stage III GBC. Mutations in ARID1A are indicative of Level 4 therapeutic implications. All ARID1A identified mutations (p.K1071*, p.R1461*, p.Q633*) were truncate nonsense oncogenic mutations. The retinoblastoma gene (Rb1) was found to be mutated in three male samples in our study corresponding to Stages II–IIIA GBC. RB1 Likely oncogenic truncating mutations (p.S567L, p.R556*, p.Y790*) result in loss of function of this gene. The AXIN1 gene was found to be mutated in two female patients (Stage IIB, Stage IIIB), with truncating mutations (p.E109* and p.Q678*).

Mutational signature analysis

APOBEC-related mutations were enriched in 24% of all samples (APOBEC enrichment score > 2) (Figure S4; Supplemental Digital Content 1, http://links.lww.com/JS9/D293). De novo signatures included spontaneous deamination of 5-methylcytosine (COSMIC_1, cosine similarity-0.831), damage by reactive oxygen species (COSMIC_18 and COSMIC_29, cosine similarity-0.864), tobacco chewing habit, exposure to tobacco mutagens (COSMIC_4, cosine similarity-0.576), and defects in polymerase POLE, in hypermutated cases (COSMIC_10, cosine similarity-0.858) (Figure S5A; Supplemental Digital Content 1, http://links.lww.com/JS9/D293). Signature patterns were plotted and annotated using the COSMIC database (Fig. 5). SBS were also identified in each sample (Figure S5B, C; Supplemental Digital Content 1, http://links.lww.com/JS9/D293). Signature 1 (COSMIC_18, COSMIC_29, COSMIC_24, and COSMIC_4) occurred in 28.8% of GBC samples, whereas Signature 2 (COSMIC_6 and COSMIC_1) correlated with age at diagnosis in 54% of samples. Signature 3, associated with smoking, was

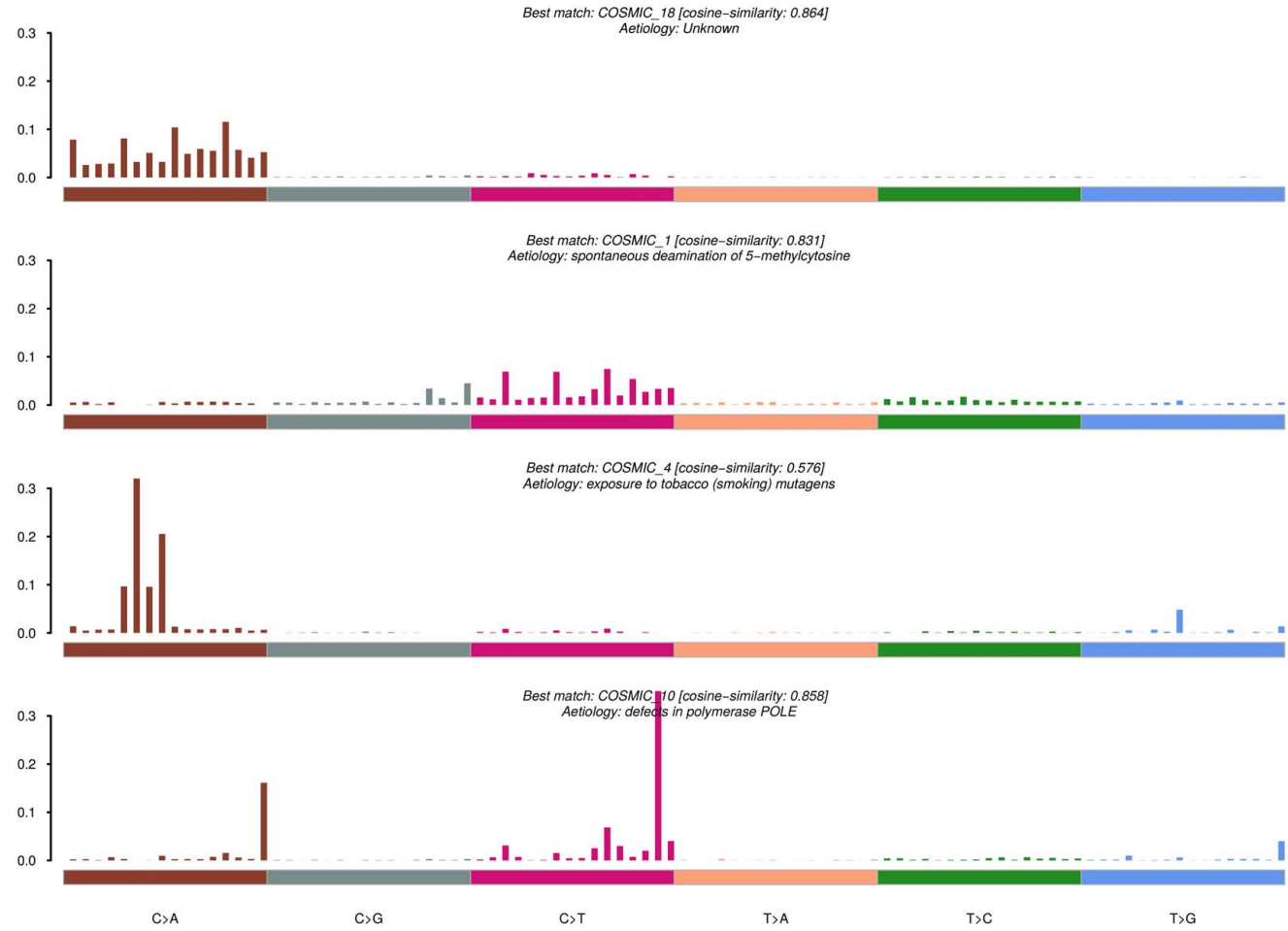

Figure 5. Mutational signatures identified in GBC cohort. The y-axis represents the exposure of 96 trinucleotide motifs to overall signatures. The plot information indicates the best match against validated COSMIC signatures, and cosine similarity value along with proposed aetiology.

Table 3

Detailed summary of COSMIC matched denovo signatures in GBC cohort.

GBC samples identified signatures	Match with COSMIC SBS signatures	Description
Signature 1 (C > A) (19 samples)	Cosmic 18(0.8)	Cosmic 18 -Mutational process underlying this signature is associated with damage by reactive oxygen species.
	Cosmic 29(0.8)	Cosmic 29-Mutational process found in cancer samples from individuals with tobacco chewing habit.
	Cosmic 4(0.7)	Cosmic 4- Associated with tobacco smoking.
	Cosmic 24(0.7)	Cosmic 24- Mutational process found in cancer samples with known exposures to aflatoxin
Signature 2 (C > T) (36 samples)	Cosmic 1(0.784)	Cosmic 1- Mutation associated with associated by the enzymatic deamination of 5-methylcytosine and correlates with age of diagnosis.
	Cosmic 6(0.7)	Cosmic 6- defective DNA mismatch repair
Signature 3 (10 samples)	Cosmic 4 (0.576)	Cosmic 4- Associated with tobacco smoking
Signature 4 (1 sample)	Cosmic 10 (0.858)	Cosmic10- Associated with generating large number of mutations and samples with these signatures have been termed as hypermutators

found in 15% of patients, and Signature 4 was associated with hypermutations in one patient (Table 3).

Tumor mutational burden analysis

TMB, indicating mutations per million bases (muts/Mb), categorizes GBC samples into high (GBC_140, GBC_157, GBC_139), intermediate (seven samples), and low TMB (Figure S6; Supplemental Digital Content 1, http://links.lww.com/JS9/D293). Comparing GBC with 33 TCGA cohorts, GBC showed low TMB (1.6 muts/mb), similar to Hepatocellular carcinoma (LIHC) (*t*-test, $P = 0.79$), and higher TMB than cholangiocarcinoma (CHOL) (Fig. 6).

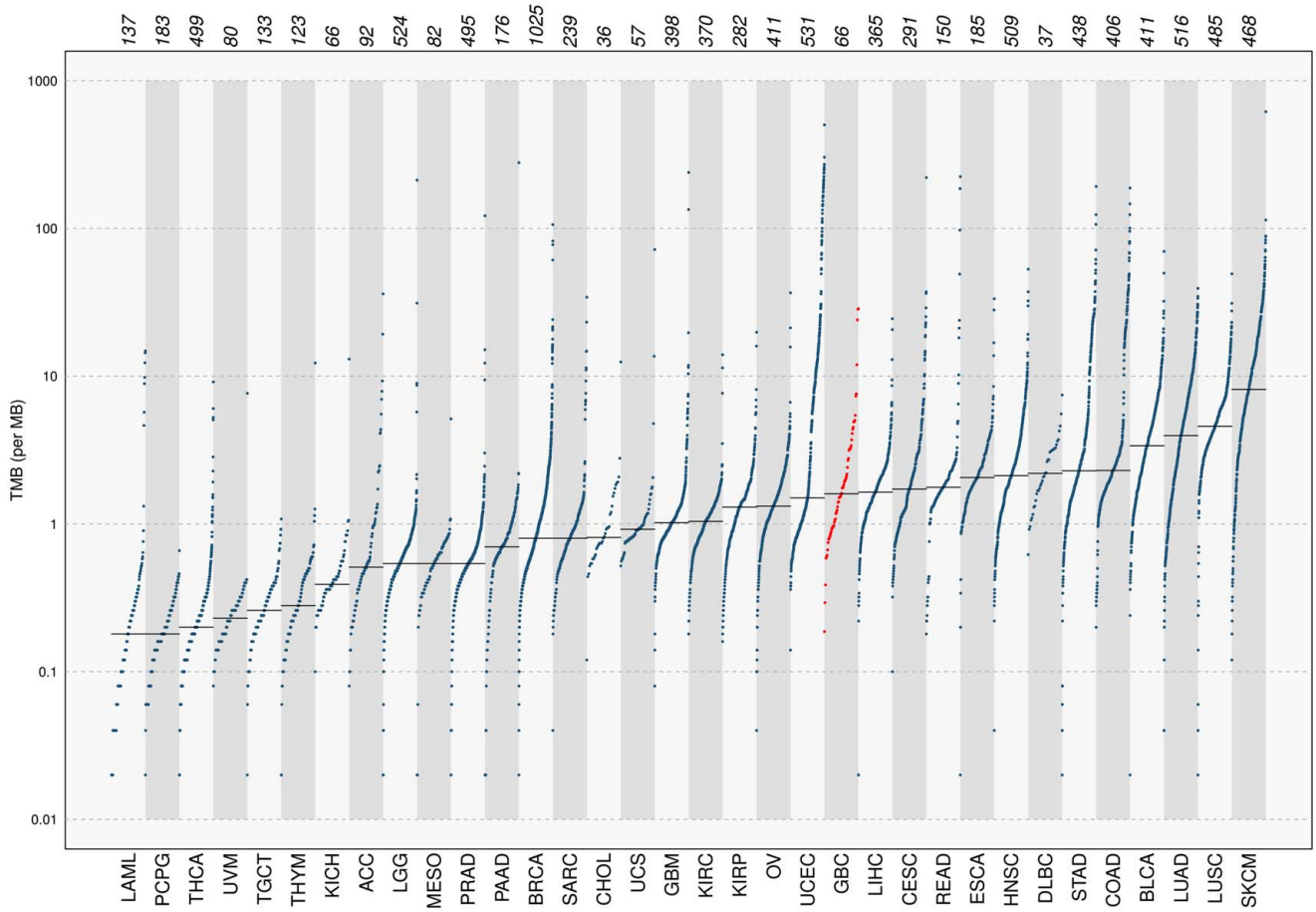

Figure 6. Prevalence of Somatic mutation burden in our population(GBC, n=66- highlighted in red color) compared to 33 TCGA cohorts. Each dot represents a single patient sample. The horizontal grey lines indicate the median number of mutations in each cancer category. Vertical axes (log scaled) showed the number of mutations per megabase.

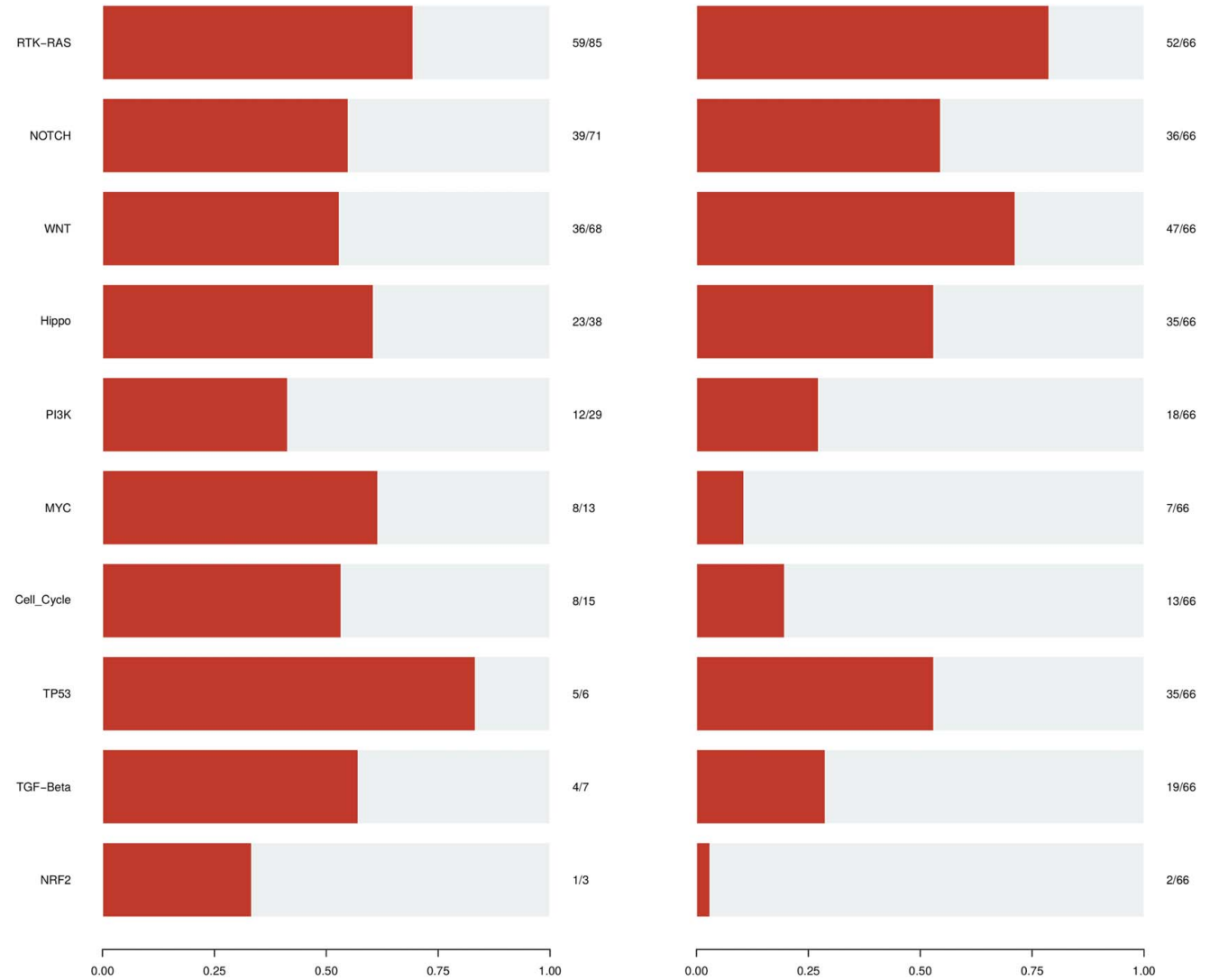

Figure 7. Overview of known altered oncogenic signaling pathways in GBC cohort. (Left) plot represents the fraction of oncogenic pathway affected and (Right) plot represents the fraction of GBC samples affected.

Mutated oncogenic signaling pathways

Genes associated with SNVs were explored across 10 key oncogenic pathways and their frequencies in affected samples are depicted (Fig. 7; Table S6; Supplemental Digital Content 2, http://links.lww.com/JS9/D294), also labeled on the signaling pathways (Fig. 8 (A-I)). The RTK-RAS pathway was enriched, with mutations detected in 52 samples (78.78%). Among these, 27 were oncogenes, while 4 exhibited tumor suppressor activity. The most frequently mutated genes were ERBB2(26.92%), ERBB3 (23.07%), ERBB4(11.53%), and KRAS (13.46%) (Figure S7A; Supplemental Digital Content 1, http://links.lww.com/JS9/D293). WNT was the second most significantly altered pathway and was mutated in 71.2% of the samples. CTNNB1 (38.29%) was the central oncogene of this pathway. AXIN1 (14.89%) was altered in 14.89% of GBC patients with tumor suppressor activity (Figure S7B; Supplemental Digital Content 1, http://links.lww.com/JS9/D293).

The Notch signaling pathway (54.54%) predominantly involved SPEN (25%), CREBBP (19.4%), and EP300 (13.9%) as the main mutated tumor suppressor genes (Figure S7C; Supplemental Digital Content 1, http://links.lww.com/JS9/D293). Mutations in the TP53 pathway accounted for 53% of cases, including TP53 (86.11%), ATM (16.66%), and CHEK2 (Figure S7D; Supplemental Digital Content 1, http://links.lww.com/JS9/D293). TP53 and ATM act as tumor suppressors, whereas MDM4 and RPS6KA3 act as oncogenes. The Hippo signaling pathway included alterations in 53.03% of GBC samples, including 16 tumor suppressor genes. Notably, HMCN1 (34.3%), DCHS2 (20%), and FAT family genes, were represented. The oncogenes involved in this pathway include the TEAD (2,3,4) family, YAP1, and HIPK2 (Figure S7E; Supplemental Digital Content 1, http://links.lww.com/JS9/D293).

The Transforming Growth Factor-β (TGF-β) pathway was mutated in 28.78% of the samples and SMAD4 (84.2%) and

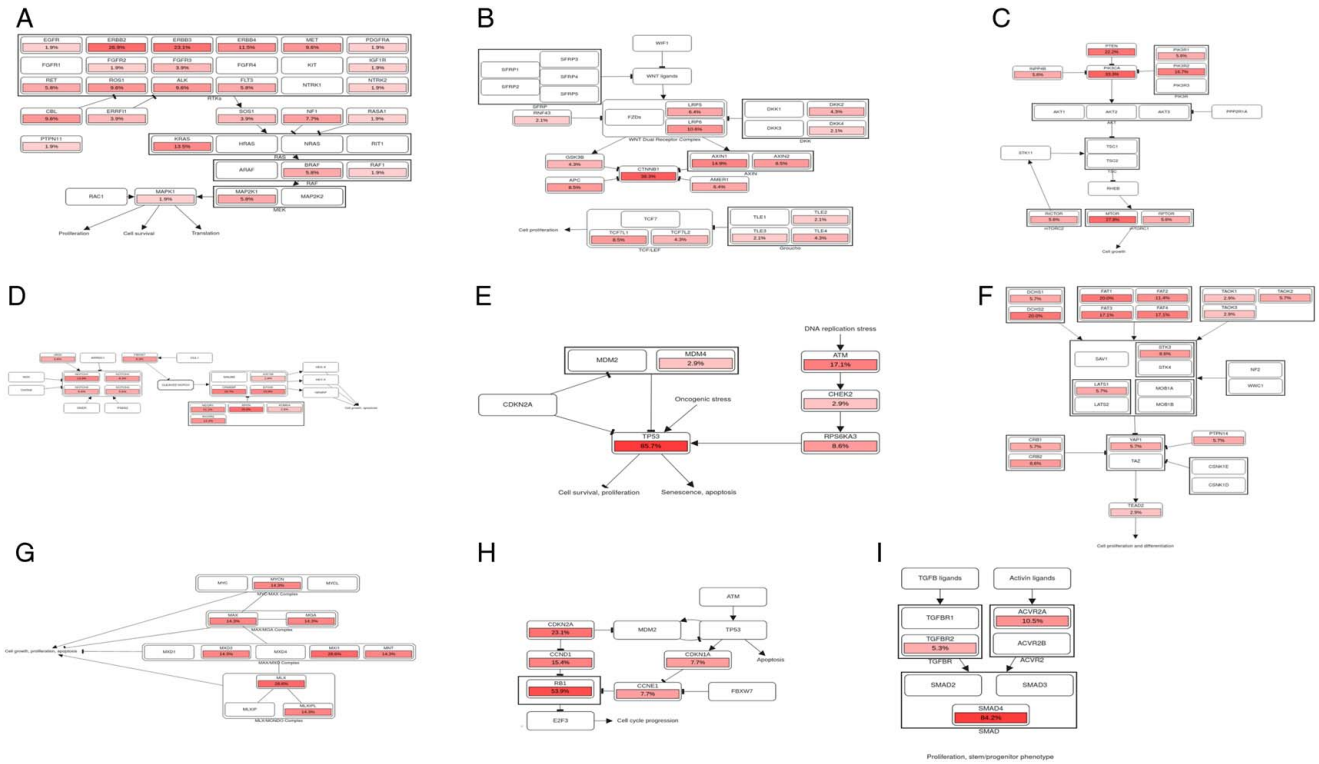

Figure 8. (A) Alteration of RTK-RAS pathway in GBC cohort. (B) Alteration of WNT pathway in GBC cohort. (C) Alteration of PI3K pathway in GBC cohort. (D) Alteration of NOTCH pathway in GBC cohort. (E) Alteration of TP53 pathway in GBC cohort. (F) Alteration of Hippo pathway in GBC cohort. (G) Alteration of MYC pathway in GBC cohort. (H) Alteration of Cell_Cycle pathway in GBC cohort. (I) Alteration of TGF-Beta pathway in GBC cohort. The color intensity represented the alteration frequency of pathway members. An arrow indicated an activation; without an arrow represented the binding activity; a bar at the end of an edge indicated an inhibitory interaction.

ACVR2A (10.5%) were the main tumor suppressor genes (Figure S7F; Supplemental Digital Content 1, http://links.lww.com/JS9/D293). The Phosphatidylinositol 3-kinase (PI3K) pathway has a mutation in 27% of GBC samples, with PIK3CA (33.3%) and MTOR (27.8%) as the main oncogenes and PTEN (22.2%) as a tumor suppressor gene (Figure S7G; Supplemental Digital Content 1, http://links.lww.com/JS9/D293). The cell cycle pathway was mutated in 19.69% of the total samples, and RB1 (53.9%) and CDKN2A (23.1%), were the main mutated tumor suppressor genes (Figure S7H; Supplemental Digital Content 1, http://links.lww.com/JS9/D293). The MYC pathway was altered in seven samples, with MAX-like protein (MLX) and MXI1 as the main mutated genes (Figure S7I; Supplemental Digital Content 1, http://links.lww.com/JS9/D293).

Discussion

In our study, we analyzed the somatic mutational landscape in 66 patients with GBC using WES, revealing a predominance of C > A and C > T mutations, which is consistent with previous GBC studies[13,25]. Missense mutations were found to be predominant in our GBC cohort. TP53, MUC16, SYNE1, SMAD4, OBSCN, ERBB2, and ERBB3 were found to be the most frequently mutated genes. Elaboration in the form of Functional analysis revealed TP53, CTNNB1, ELF3, SMAD4, ERBB2, ARID1A, AXIN1, and RB1 as potential driver genes in 86% of samples. Considering the genes with oncogenic and clinically significant effects in at least two patients, our study identified the major role of eight genes- TP53, SMAD4, ERBB3, KRAS, ARID1A, PIK3CA, RB1, and AXIN1.

Our analysis revealed significant TP53 alterations, which is consistent with prior WES studies on GBC. Previous studies have found TP53 mutations in 27–70% of gallbladder carcinomas, emphasizing the relevance of this gene in GBC pathology[55]. All TP53 missense and nonsense variants found in our investigation were related to loss of function and had a prognostic degree of significance (level Px1). TP53 plays a pivot role in tumor suppression and regulates various cellular processes such as – apoptosis, immune response modulation, and autophagy[56]. During DNA damage, wild-type p53 prevents defective cell proliferation and cancer development, whereas TP53 mutations promote DNA damage and disease progression[57]. In our GBC cohort, specific TP53 mutations such as p.K132T have been reported in glioblastoma[58], p.M133T associated with an unregulated inflammatory response that has been studied in breast cancer[59], p.H179R mutation related to downregulation of the genes associated with cell cycle arrest and apoptosis[60], and p. E285K associated with loss of function[61]. The P.G245S mutation was found in cell lines that lacked TP53 expression and did not mediate transcriptional activity[62]. Another TP53 mutation p.S127F, has been identified as significant hotspot in xeroderma pigmentosum[63]. These alterations result in loss of tumor suppressor activity and aggravate the carcinogenic process in GBC patients[64].

SMAD4 mutations have emerged prominently after TP53 alterations, indicating their sequential involvement in GBC

progression. SMAD4 variants primarily disrupt the TGF-β signaling system, resulting in loss of function through missense and nonsense variants. Activated TGF receptors phosphorylate SMADs, which cause them to translocate to the nucleus and enhance transcriptional activity[65]. Initially, during normal pathway regulation, the associated TGF receptors have tumor suppressor activity but dysregulation by loss of function results in unregulated growth and progression of cancer[66]. Specific SMAD4 mutations in our study, such as p.D537Y, p.G352V reduce antiproliferative TGF-mediated transcriptional activity and have been reported in colorectal cancer[67]. Notably, our study identified the p.D493N mutation located in the MH2 domain, which has been previously reported in GBC[11]. Additionally, p.E330K, p.W509R, and p.R361H mutations were associated with decreased SMAD4-mediated transcription[68], decreased BMP signaling[69] and decreased antiproliferative mediated transcription[70].

ERBB3 showed significant missense mutations associated with gain of function in the RTK-RAS pathway. ERBB3 forms dimers with ERBB2 to exert its effect. Although frequent ERBB2 mutations were observed in our study, their significance regarding cancer activity was uncertain. Previous studies have reported both ERBB2 and ERBB3 alterations in 12% of GBC cases[11]. The identified ERBB3 oncogenic mutations in GBC cohort include p.G284R, p.E298G reported in cholangiocarcinoma, breast, and colon cancer[71], p.V104L found in bladder and colon cancer[72], p.P262H, documented in colon and ovarian cancer[73] and p.V104M mutations, identified in cholangiocarcinoma, breast cancer, and endometrial cancer[74]. KRAS mutations were significantly mutated from prognostic level 2 to 4 in the GBC cohort, with diagnostic relevance and missense mutations. In previous GBC studies, KRAS mutations were reported in 4–13% of GBC[75]. RTKs orchestrate essential signaling cascades involved in key cellular processes such as proliferation, differentiation, survival, and migration[76]. Alterations in RTKs are frequently observed as contributing factors in the progression of numerous cancers and uncontrolled cell proliferation[77]. The activation of RTKs by external ligands promotes downstream signaling mediated by RAS proteins. These proteins further relay signals for processes, such as anti-apoptosis and cell regulation. Specific KRAS mutations, such as p.G12V, p.G12D were found to be prevalent in lung and pancreatic cancer[78], and p.V14I mutation is linked to Noon syndrome[79].

Next, PIK3CA missense mutations were prevalent with level 1 therapeutic significance in our GBC cohort. PI3K signaling dysregulation has been investigated in previous GBC studies emphasizing cancer cell proliferation and metastasis with PIK3CA gain-of-function and loss of PTEN mutation[80]. Approximately 8 and 50% of human GBC cases are associated with dysregulated PI3K signaling in GBC pathogenesis[81]. Mutations such as p.R38H, p.E545K mutation has been reported in glioblastoma[82], and p.E365K is linked to endometrial cancer[83]. ARID1A truncating mutations have level 4 implications underscoring their role in pathogenesis[21,23]. Previous studies have supported the role of ARID1A nonsense mutation in the proliferation of cancers, which is approximately prevalent among 13% of patients with GBC and has the worst prognosis correlation in BTC[84,85].

RB1 truncating mutations lead to loss of function and truncation, without diagnostic or prognostic implications in the GBC cohort. RB1 mutations in the cell cycle pathway have been reported in a previous GBC study[12]. The pathway plays a major role in the progression and therapeutic response of different cancers[86]. AXIN1 associated truncating mutations had oncogenic activity but without any diagnostic or prognostic prevalence in the cohort. AXIN1 is a crucial component of the β-catenin destruction complex in the WNT pathway, and plays a pivotal role in regulating cell proliferation and differentiation. AXIN1 mutations have been detected in a various malignancy, including liver cancers[87]. Dysregulation of WNT system-associated genes promotes tumor growth and angiogenesis, and AXINI mutations linked to this pathway have been identified in a prior GBC study[88].

Our analysis demonstrated a link between COSMIC signatures 1 and 6, which correlated with age at diagnosis and faulty DNA mismatch repair mechanisms, consistent with prior studies on GBC[14,16,17]. Furthermore, the COSMIC signatures of 18 and 29 mutations were detected in patients with a history of tobacco chewing, highlighting the clinical importance of addictive practices in the development of GBC.

Conclusion

Our findings revealed recurrent mutations, including possibly pathogenic and carcinogenic variations. The eight discovered genes – TP53, SMAD4, ERBB3, KRAS, ARID1A, PIK3CA, RB1, and AXIN1 – all exhibited important roles in driving GBC development – had a significant collective impact on GBC development in our cohort. These mutations have great potential for inclusion in the GBC diagnostic gene panels. Notably, TP53 and SMAD4 ERBB3 were identified as the most frequently altered genes, especially in advanced GBC cases. Furthermore, our research discovered mutational signatures associated with age and tobacco addiction, which are more common in patients diagnosed at a later stage. In our study, half of GBC patients had a smoking or tobacco addiction, indicating a clinical correlation with our findings. The RTK-RAS and WNT pathways were the most frequently altered oncogenic signaling pathways in our analysis, emphasizing GBC's complexity and tumor heterogeneity. Our findings support the importance of personalized therapy based on individual genomic landscapes. However, further research is required to confirm our findings and determine their functional significance in tumor growth and progression. Integrating multiomics data with larger cohort studies has the potential to expand our understanding of disease biology, clinical management, and improving patient outcomes.

Ethical approval

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of All India Institute of Medical Sciences for a study involving humans. Ref No.-IEC-32/09.03.2018, OP-5/09.03.2018.

Consent

We received informed consent from all the cases involved in the study.

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Author contribution

P.T.: conceptualization, supervision, and writing – review and editing; S.A.: design, conceptualization, resources, WES experiments, data curation, data analysis, writing – original draft, and writing – review and editing; D.P.: conceptualization, design, review, and editing; Rahk, H.G.: writing – review and editing; N. R.: resource and initial WES experiment; N.R.D., S.K., and S.S.S.: resources; U.A., S.H., S.S.S., P.D., P.S., S.S., and R.R.: writing – review and editing; G.K.R., T.K., and R.S.D.: project administration, writing –review and editing. All authors have read and approved the final manuscript.

Conflicts of interest disclosure

The authors declare that they have no financial conflict of interest with regard to the content of this report.

Guarantor

Pranay Tanwar.

Data availability statement

The raw data generated during the current study is available in the NCBI repository with BioProject ID - PRJNA1058876 and PRJNA1049991. The data is not publicly available due to restrictions. Derived data supported the findings of this study are available from the corresponding author on request.

Presentation

None.

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