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Integrated co-mutation profiling reveals a novel genomic classification for prognostication and tailored therapy in biliary tract cancer

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Abstract

Biliary tract cancers (BTCs) exhibit substantial genomic heterogeneity, which is further compounded by the presence of co-mutations, thereby increasing therapeutic complexity. Utilizing a large discovery cohort of 2,378 patients and an independent immunochemotherapy (ICT) cohort of 59 patients, this study investigates the patterns of co-mutation in BTCs and their clinical implications. We identified co-mutation features with potential relevance for guiding targeted therapeutic strategies. Furthermore, we delineated deleterious co-mutations (DCMs) and deleterious single mutations (DSMs) associated with adverse clinical outcomes. By integrating DCMs and DSMs, we classified BTCs into three genomic subtypes: wild-type (WT), single-hit (SH), and multi-hit (MH). The MH subtype is characterized by a high co-mutation burden and specific mutational signatures. Despite having a high prevalence of microsatellite instability and abundant actionable alterations, this subtype shows a significantly worse response to ICT. It is also associated with shorter overall survival. In contrast, the WT subtype exhibits the best outcomes. Our findings provide a molecular framework for stratifying BTC patients. MH tumors might be prioritized for genotype-matched targeted therapy, although direct comparative evidence is still lacking.

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Introduction

Biliary tract cancers (BTCs), which include cholangiocarcinoma (CHOL) and gallbladder carcinoma (GBC) based on anatomical location, have shown a steadily increasing incidence in recent years[1]. These malignancies are often insidious in onset, with the majority of patients diagnosed at an advanced stage[2]. Their aggressive biology, marked molecular heterogeneity, and strong drug resistance contribute to exceptionally challenging treatment and very low five-year survival rates[3].

In recent years, immune checkpoint inhibitors (ICIs) have represented a major breakthrough in BTC treatment. The phase 3 randomized controlled trial TOPAZ-1 provided the first key evidence for a new combination therapy[4]. It showed that adding durvalumab to standard chemotherapy (gemcitabine and cisplatin) significantly improved patient outcomes. Compared to chemotherapy alone, the combination therapy led to a median overall survival (OS) of 12.9 months vs 11.3 months. Progression-free survival (PFS) was also better, at 7.2 months compared to 5.7 months. Additionally, the objective response rate (ORR) increased from 19% to 27%. A second major trial, the similarly designed phase 3 KEYNOTE-966 study was subsequently conducted[5]. It successfully met its primary endpoint by demonstrating that adding pembrolizumab to chemotherapy extended median OS. The improvement was from 10.9 to 12.7 months. However, the addition of pembrolizumab did not improve other key outcomes. PFS remained similar (6.5 months vs 5.6 months), and the ORR was identical in both groups at 29%. Moreover, the absolute survival benefit in both trials remained modest, and more than 70% of patients derived no response to the combination therapy.

A key strategy for improving therapeutic efficacy involves selecting optimal patient subgroups or excluding less responsive ones using molecular biomarkers. However, the KEYNOTE-966 trial indicated that the classical immunotherapy biomarker PD-L1 expression showed no clear correlation with survival benefit[5]. Furthermore, the relationship between tumor mutational burden (TMB) and immunotherapy efficacy in BTCs remains controversial[6], and microsatellite instability (MSI) is rare in this cancer type[7]. Nevertheless, specific genetic alterations may critically influence immunotherapy outcomes. A phase II study of first-line camrelizumab combined with gemcitabine/oxaliplatin (GEMOX) in advanced BTCs reported an ORR of 54% and a median OS of 12.1 months[6]. Subsequent analysis identified an 11-gene mutational signature that stratified BTC into 'mutant' and 'wild-type' subgroups, with significantly worse immunotherapy outcomes observed in the mutant group[8]. Another recent retrospective study of 125 advanced BTC patients treated with first-line anti-PD-1/PD-L1 plus chemotherapy found that responders had higher frequencies of *TP53*, *BRCA2*, and cytokine-pathway mutations, whereas *KRAS* and *ARID1A* mutations were associated with poorer efficacy. Combining CXCL9 and CTLA4 expression levels further improved prognostic stratification[9]. However, these findings explain only a fraction of treatment outcomes, likely because concurrent mutations exert additional effects.

Co-occurring mutations further enhance molecular heterogeneity in malignancies. In non-small cell lung cancer (NSCLC), for example, concurrent *TP53* and *STK11* mutations subdivide *KRAS*-mutant NSCLC into two immunologically distinct subtypes. The *KRAS/TP53* group shows activated immune-cell infiltration and elevated PD-L1 expression, whereas the *KRAS/STK11* group exhibits the opposite profile[10]. Accordingly, *KRAS/TP53* NSCLC benefits more from immunotherapy[11], while *KRAS/STK11* tumors not only respond poorly but may also experience reduced survival[12,13]. The impact of co-mutations on the immune microenvironment and

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tumor progression has also been reported in other cancers, including colorectal[14], pancreatic[15], breast[16], and prostate cancers[17], and extends to targeted therapies such as anti-EGFR tyrosine kinase inhibitors (TKIs)[18], anti-HER2 monoclonal antibodies[19], and Poly ADP-ribose polymerase (PARP) inhibitors[17]. However, most studies have focused on co-mutations in a limited set of classic oncogenes and tumor suppressor genes, leaving the clinical implications of other concurrent mutations largely unexplored.

The effect of co-mutations on immunochemotherapy (ICT) outcomes in advanced BTCs remains unclear. This study aims to define the landscape and characteristics of co-mutations in BTCs, identify co-mutation patterns associated with ICT response, and integrate prognostically significant single mutations to establish a novel genomic classification system for BTCs. By leveraging the molecular features of each subtype, we propose tailored treatment strategies. These insights are expected to improve therapeutic outcomes for patients with advanced BTCs.

Methods

Patients

This study included two cohorts. A large discovery cohort of 2,378 patients with advanced BTC and known MSI status was used to characterize co-mutation profiles and molecular features. These were routine clinical cases collected from multiple centers between January 2018 and April 2023. A smaller ICT cohort with complete outcome data was assembled for efficacy and prognostic analysis, including 34 patients from the phase II clinical trial NCT03486678 who received first-line camrelizumab plus GEMOX[8], and 25 real-world patients who received similar ICT. Objective responses were assessed per RECIST 1.1, and OS was defined as the time from treatment initiation to death from any cause. All patients had pathologically confirmed BTC, no prior antitumor therapy before sampling, and sufficient qualified tissue for sequencing. Exclusion criteria included a history of other malignancies, multiple primary tumors, and combined hepatocellular-cholangiocarcinoma. Genomic data are available in the Genome Sequence Archive for Humans (https://ngdc.cncb.ac.cn/gsa-human) under project PRJCA031321, and dataset HRA008984. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. Written informed consent was obtained from all participants.

Sequencing and genomic analysis

Targeted sequencing was performed in laboratories accredited by the Clinical Laboratory Improvement Amendments (CLIA) and the College of American Pathologists (CAP), using clinically validated gene panels[8,20] (Supplementary Table S1). Detailed sequencing and variant-calling methods have been described previously[20]. MSI status was determined with an in-house Python pipeline[21]. This study focused on somatic non-synonymous variants (SNVs) that are known or predicted to be functional[22]. TMB was defined as the total number of such SNVs[23]. The Between-Within (Bewith) method was used to analyze co-mutations and mutually exclusive mutations[24]. The former is defined as a set of genes that are mutated concurrently in the same tumor sample at a frequency significantly higher than expected by chance, while the latter is defined as a set of genes that are rarely or never concurrently mutated in the same tumor sample. The total number of co-mutations present in each sample was defined as the co-mutation burden.

PD-L1 assay

PD-L1 expression was assessed by immunohistochemistry using the Dako PD-L1 IHC 22C3 pharmDx kit and Autostainer Link 48 system (Agilent Technologies). Expression levels were evaluated using the Tumor Proportion Score (TPS), with positivity defined as TPS ≥ 1%.

Statistical analysis

Group comparisons were performed using the χ^2 test, Fisher's exact test, Student's t-test, or Wilcoxon/Mann–Whitney test, as appropriate. Receiver operating characteristic (ROC) curves and area under the curve (AUC) were used to evaluate the diagnostic value of co-mutational load for MSI. Survival probabilities were estimated using Kaplan–Meier curves and compared with the log-rank test. Multivariate analysis used Cox proportional hazards regression. Least absolute shrinkage and selection operator (LASSO) regression was applied to identify gene co-mutations or single mutations with the strongest prognostic impact. Statistical significance was set at $p < 0.05$ (two-tailed). Analyses were conducted using SPSS 23.0, R 4.3.2, and relevant Bioconductor packages.

Results

Patient characteristics

In the discovery cohort of 2,378 patients (Supplementary Table S2), CHOL was predominant (1,702 cases, 71.6%), while GBC accounted for 676 cases (28.4%). Younger patients (< 65 years) were more common (1,462, 61.5%), and 916 (38.5%) were ≥ 65 years. The male-to-female ratio was nearly balanced (1,253 males, 52.7%; 1,125 females, 47.3%). MSI tumors were identified in only 54 patients (2.3%). In the ICT cohort (Supplementary Table S3), CHOL again predominated (62.7%), younger patients were the majority (62.7%), and males outnumbered females (61% vs 39%). MSI status was available for 48 patients, of whom three were MSI (5.1%). A total of 46 patients (78%) received first-line ICT, and 13 (22%) were treated in the second-line setting.

Co-mutation characteristics

We first characterized co-mutation patterns in the discovery cohort. *TP53* and *KRAS* mutations were highly prevalent (Fig. 1a), likely reflecting the advanced stage of the cohort. *TP53* displayed frequent co-mutations and mutual exclusivity, while *KRAS* showed the highest number of mutually exclusive events. *TGFBR2*, *ARID1A*, *ARID1B*, *KMT2C*, *LRP1B*, *PIK3CA*, *KMT2D*, and *PBRM1* were each enriched for co-mutations (Fig. 1b). At the pathway level, RTK-RAS alterations were significantly co-occurrent with *TGF-beta* mutations but mutually exclusive with *PI3K* and *TP53* pathway lesions; alterations in other pathways tended to co-occur (Fig. 1b).

Co-mutation burden was strongly correlated with TMB ($R = 0.82$, $p < 0.001$; Fig. 1c). MSI-high tumors had a significantly higher co-mutation load than MSS tumors (Fig. 1d), and co-mutation burden alone predicted MSI status with an AUC of 0.940 (Fig. 1e). Using the Youden index to dichotomize co-mutation burden and TMB, the double-high group had an MSI frequency of 60%, significantly higher than the 1.1% observed when only one metric was high (Fig. 1f).

Fig. 1 Characteristics of co-mutations in the discovery cohort. (a) Waterfall plot illustrating the frequency and distribution of the top 20 most frequent mutations. (b) Analysis of mutual exclusivity and co-occurrence among the top 20 mutations, including their relationships within 10 major oncogenic signaling pathways. *TP53* displayed frequent co-mutations and mutual exclusivity, while *KRAS* showed the highest number of mutually exclusive events. (c) Pearson correlation between co-mutation burden and total mutation burden (p < 2.2e-16). (d), Comparison of co-mutation burden between microsatellite-instable (MSI) and microsatellite-stable (MSS) tumors. (e), Receiver operating characteristic (ROC) curve and area under the curve (AUC) for predicting MSI status based on co-mutation burden (the Youden index is 4.5). (f) Using the Youden index derived from the ROC curve, both co-mutation and total mutation burdens were dichotomized into high and low groups. The incidence of MSI across the resulting combined subgroups is shown. The double-high group had an MSI frequency of 60%, significantly higher than the 1.1% observed when only one metric was high. CHOL, cholangiocarcinoma; GBC, gallbladder cancer; ROC, receiver operating characteristic; AUC, area under the curve.

Characteristics of co-occurring actionable alterations

BTCs are characterized by a high prevalence of actionable alterations[25], yet the patterns of co-occurrence and mutual exclusivity among these mutations remain poorly understood. In our discovery cohort, 68% of tumors harbored at least one actionable lesion; the five most frequent were *KRAS* mutation, *PIK3CA* mutation, *ATM* mutation, *NF1* mutation, and *ERBB2* amplification (Fig. 2a). The actionable landscapes of CHOL and GBC differed significantly: *KRAS* and *IDH1*/*IDH2* mutations and *FGFR2* fusions/rearrangements were enriched in CHOL, whereas *ERBB2* amplification/mutation and *PIK3CA* or *ATM* mutations were more common in GBC (Fig. 2b). We therefore analyzed mutational interactions separately in CHOL and GBC. In CHOL, *KRAS* mutation showed negative selection, being

Fig. 2 Actionable alterations and their mutual exclusivity and co-occurrence patterns. (a) Waterfall plot displaying the frequency and spectrum of actionable alterations. (b) Comparison of actionable alteration frequencies between CHOL and GBC. The actionable landscapes of CHOL and GBC differed significantly. Patterns of mutual exclusivity and co-occurrence among actionable alterations in (c) CHOL, and (d) GBC, respectively. In CHOL, *KRAS* mutation showed negative selection, being significantly mutually exclusive with most other actionable alterations. In contrast, GBC was characterized by *PIK3CA* and *IDH2* mutations co-occurring with multiple other actionable lesions. AMPL, amplification; FS, fusion; RA, rearrangement.

significantly mutually exclusive with most other actionable alterations (Fig. 2c). In contrast, GBC was characterized by *PIK3CA* mutations co-occurring with multiple other actionable lesions (Fig. 2d), which may inform precision therapy strategies.

Drugs targeting *ERBB2* amplification and *FGFR2* fusion/rearrangement have been approved for BTCs[26,27]; however, patients with *ERBB2* or *FGFR2* mutations often receive these agents with reduced efficacy. Co-mutation profiling revealed that *ERBB2*-amplified tumors had a significantly higher rate of concurrent *TP53* mutations than *ERBB2*-mutant tumors (81.0% vs 61.4%, $p < 0.001$; Supplementary Fig. S1a, S1b), while co-occurrence of other gene mutations was significantly lower (Supplementary Fig. S1c). In *FGFR2* fusion/rearrangement tumors, *BAP1* was the most frequent co-alteration, occurring more often than in *FGFR2*-mutant cases (35.9% vs 12.8%; Supplementary Fig. S1d–S1f). In contrast, *FGFR2*-mutant tumors most commonly had concurrent *TP53* mutations (43.6%; Supplementary Fig. S1e), and the prevalence of *TP53* and other gene mutations was significantly higher than in the fusion/rearrangement subgroup (Supplementary Fig. S1f). This data suggests that *ERBB2* and *FGFR2* mutations are associated with a more complex co-mutational landscape than amplifications or rearrangements, potentially explaining the reduced response to targeted therapies.

Co-mutations associated with prognosis

We next evaluated the relationship between co-mutations and prognosis in the ICT cohort. Univariate Cox regression identified 79 deleterious co-mutations (DCMs) significantly associated with shortened OS ($p < 0.05$; Supplementary Table S4, Fig. 3a). LASSO regression refined these to the 16 most influential events (Supplementary Table S5, Fig. 3b). Patients with any of these high-risk DCMs had a median OS of only 5.6 months, significantly worse than the 11.8 months in those without (HR = 8.44, 95% CI: 3.97–17.93; Fig. 3c). Multivariate Cox analysis adjusted for patient source (trial vs real-world), tumor subtype (CHOL vs GBC), age (≥ 65 vs < 65 years), sex (male vs female), and treatment line (first-line vs second-line), confirmed that DCMs were an independent predictor of poor OS (HR = 9.60, 95% CI: 4.21–21.85, $p < 0.001$; Fig. 3d). Among 33 patients with response data, the ORR was only 25% in the DCM subgroup, significantly lower than the 58.6% in those without DCMs (Fig. 3e).

Using a similar approach, we refined 10 deleterious single mutations (DSMs; $p < 0.05$; Supplementary Table S6, Fig. S2a) to the six with the strongest impact (Supplementary Table S7, Fig. S2b). Intersection of genes involved in DCMs and these high-risk DSMs yielded five shared genes: *ARID1A*, *MED12*, *TNFAIP3*, *PTEN*, and *SMO* (Supplementary Fig. S2c). For DCMs involving these genes, their adverse prognostic effect was partly attributable to the single mutations. For all other co-mutation pairs, neither single mutation alone influenced outcome, but their combination was prognostically significant (Supplementary Fig. S2d). Whether this reflects biological interaction requires further investigation.

A novel classification system based on DSMs and DCMs

We observed a strong dose-response relationship between the number of DSMs and outcomes: more mutations correlated with worse prognosis. No sample had more than two such mutations, suggesting a potent cumulative effect (Fig. 4a). Based on this, we developed a novel genomic classification for BTCs integrating DSMs and DCMs: single-hit (SH) subtype (one DSM only); multi-hit (MH) subtype (≥ 1 DSM and/or ≥ 1 DCM); and wild -type (WT) subtype (neither).

Outcomes differed significantly among the three groups: WT tumors had the best survival, SH intermediate, and MH the worst (Fig. 4b). Stratified analysis confirmed that MH status remained a robust adverse factor across all clinically relevant subgroups (Fig. 4c). ORR mirrored survival: 66.7% for WT, 42.9% for SH, and only 20% for MH tumors (Fig. 4d).

Dissection of the MH category (Supplementary Fig. S3a) showed that most cases (61.5%) had only DCMs, 30.8% had both DSMs and DCMs, and one patient (7.7%) had two DSMs. Survival did not differ among these patterns (Supplementary Fig. S3b).

Characteristics of the three newly defined subtypes

We next assessed the frequency and characteristics of the three subtypes in the large cohort. Among 2,378 patients, 10.6% were MH, 17.6% SH, and 71.8% WT (Fig. 5a). The MH group comprised 80.6% with only DCMs, 15.4% with both DCMs and DSMs, and only 10 cases (4%) with > 1 DSMs and no DCMs (Fig. 5b).

Despite having the worst prognosis, MH tumors had the highest MSI rate (13.9%), followed by SH (2.4%) and WT (0.5%) (Fig. 5c). In the ICT cohort, only three patients were MSI: one MH and one SH died at 6 months, while the WT patient was alive at last follow-up (Supplementary Fig. S4a), suggesting that deleterious mutations may diminish ICT benefit even in MSI tumors. Among MSS patients, the three subtypes still stratified outcomes significantly (Supplementary Fig. S4b).

Mutational landscapes differed markedly among subtypes (Fig. 5d–f). *TP53* was most frequent in MH (74%), *ARID1A* in SH (77%), and *KRAS* in WT (31%). Most genes were mutated at higher rates in MH than in SH, or WT (Supplementary Fig. S5a, b). Notably, *PIK3CA*, *NF1*, and *ATM* were more frequently mutated in MH than in SH; combining mutation status of these three genes correctly distinguished > 84% of MH from SH cases (Fig. 5g). These features facilitate clinical implementation of the classification.

Enrichment analysis of differentially mutated genes among the three subtypes

To explore the biological significance of characteristic mutations in each subtype, we compared mutation frequencies pairwise among the three subtypes (Supplementary Tables S8–S10, Fig. S5), and performed enrichment analysis of significantly differential mutations using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, Reactome Pathways, and biological processes of Genome Ontology (GO) (Supplementary Tables S11–S13). Compared with WT, mutations enriched in the MH subtype were associated with signaling pathways related to drug resistance (e.g., platinum drug resistance), immune dysregulation (e.g., human T-cell leukemia virus 1 infection), and proliferative activity (e.g., ERBB2 signaling) (Supplementary Fig. S6a, S6b). Furthermore, compared with both WT and SH, MH-enriched mutations were linked to biological functions such as epigenetic regulation (e.g., chromatin remodeling), growth and development (e.g., developmental growth), and angiogenesis (e.g., blood vessel morphogenesis) (Supplementary Fig. S6c, S6d). SH-enriched mutations were associated with homologous recombination deficiency and cell cycle regulation compared with WT (Supplementary Fig. S6e, S6f). These findings suggest potential avenues for personalized therapy in MH and SH subtypes.

Mutation signatures in the three subtypes

By comparing against the Catalogue of Somatic Mutations in Cancer (COSMIC) signature database, we found that the MH subtype was dominated by defective DNA mismatch repair (cosine

Fig. 3 Prognostic impact of co-mutations. (a) Univariate Cox regression analysis identifying co-mutations significantly associated with prognosis (top 20 shown). (b) Selection of detrimental co-mutations (DCMs) with the strongest prognostic influence using LASSO regression. (c) Kaplan-Meier survival curves comparing patients with versus without DCMs. (d) Multivariate Cox regression evaluating the independent prognostic value of DCMs confirmed that DCMs were an independent predictor of poor OS. (e) Objective response rate (ORR) among 33 patients with available treatment-response data, stratified by the presence of DCMs. The ORR was only 25% in the DCM subgroup, significantly lower than the 58.6% in those without DCMs. mOS, median overall survival; HR, hazard ratio; CI, confidence interval; RW, real-world; 1st, first-line; 2nd, second-line. LASSO: Least absolute shrinkage and selection operator.

similarity = 0.921) and APOBEC cytidine deaminase activity (C > T; 0.699) (Supplementary Fig. S7a, S7b). The SH subtype also showed the mismatch repair signature (0.885) and an additional signature not currently in COSMIC (0.643) (Supplementary Fig. S7c, S7d). The WT subtype carried a broader range of signatures, including spontaneous/enzymatic deamination of 5-methylcytosine (0.895), APOBEC (0.781), alkylating agent exposure (0.465), and prior platinum chemotherapy (0.682) (Supplementary Fig. S7e, S7f). Consistent with this, APOBEC-enriched samples were most common in the MH subtype (40.5%), followed by SH (29.9%) and WT (28.2%) (Supplementary Fig. S8a–S8d). These findings further highlighting mutational heterogeneity among subtypes.

Fig. 4 Genomic subtyping based on integrated DSMs and DCMs. (a) Survival analysis stratified by the number of detrimental single mutations (DSMs). A strong dose-response relationship was observed between the number of DSMs and outcome. (b) Survival analysis according to the proposed genomic subtypes. Outcomes differed significantly among the three groups: WT tumors had the best survival, SH intermediate, and MH the worst. (c) Stratified analysis confirmed that MH status remained a robust adverse factor across all clinically relevant subgroups. (d) ORR across genomic subtypes. WT, wild-type; SH, single-hit; MH, multi-hit.

Actionable targets in the three subtypes

We examined differences in actionable targets among the subtypes. Up to 92.5% of MH tumors harbored ≥ 1 actionable alteration; the most frequent were *NF1* (38.1%), *PIK3CA* (28.2%), and *ATM* (27.4%) mutations (Fig. 6a). Most actionable alterations occurred significantly more often in MH than in SH or WT, including mutations in *ATM*, *BRCA1*, *BRCA2*, *ERBB2*, *FGFR1*, *FGFR2*, *MAP2K1*, *NF1*, *PIK3CA*, and *ERBB2* amplification. Conversely, *FGFR2* fusions/

Fig. 5 Characteristics of the genomic subtypes. (a) Distribution of the three subtypes within the discovery cohort. (b) Composition of the MH subtype. (c) Prevalence of MSI across the three subtypes. MH tumors had the highest MSI rate (13.9%), followed by SH (2.4%), and WT (0.5%). Waterfall plots showing the top 20 mutations in the MH (d), SH (e), and WT (f) subtypes. (g) Proportion of MH and SH subtypes among tumors with *PIK3CA*/*NF1*/*ATM* mutations versus wild-type. Combining mutation status of *PIK3CA*, *NF1*, and *ATM* correctly distinguished > 84% of MH from SH cases. MDSM, multiple DSMs.

Group	TPS≥1 (NO)	Total (NO)
WT	5	22
SH	1	5
MH	0	4

Fig. 6 Actionable alterations in the MH subtype. (a) Waterfall plot of actionable alterations. (b) Mutual exclusivity and co-occurrence among actionable alterations. *NF1* mutation was mutually exclusive with *ERBB2*, *ATM*, and *PIK3CA* mutations. *ATM* mutation/amplification co-occurred with *ERBB2* mutation/amplification, and *IDH2* mutation co-occurred with *MET* mutation. (c) PD-L1 positivity rates across the three subtypes. The positivity rate (TPS ≥ 1) was 0% in MH, markedly lower than in SH (20.0%), and WT (22.7%).

rearrangements, *IDH1* mutation, *KRAS* mutation, and *MDM2* amplification were less common in MH (Table 1).

Interaction analysis within MH tumors showed that NF1 mutation was mutually exclusive with *ERBB2*, *ATM*, and *PIK3CA* mutations. *ATM* mutation/amplification co-occurred with *ERBB2* mutation/amplification, and *IDH2* mutation co-occurred with *MET* mutation (Fig. 6b). Among 31 samples with PD-L1 data, the positivity rate (TPS ≥ 1) was 0% in MH, markedly lower than in SH (20.0%) and WT (22.7%) (Fig. 6c).

These findings suggest that although MH tumors are poorly suited to ICT, they harbor numerous actionable targets, indicating that matched targeted therapy may be a superior approach, though overlapping interactions among alterations must be considered.

Discussion

This study provides a comprehensive analysis of co-mutation patterns in advanced BTCs and their impact on ICT outcomes. We identified 16 DCMs strongly associated with poor survival and response, and integrated these with DSMs to establish a novel genomic classification system for BTCs. The classification, including WT, SH, and MH subtypes effectively stratified patients into distinct prognostic subgroups with significant differences in OS and ORR. These results highlight the critical role of co-mutational landscapes in shaping therapeutic efficacy and prognosis in BTC.

The high prevalence of *TP53* and *KRAS* mutations in our cohort aligns with previous reports in advanced BTCs[25,28]. Beyond single-gene alterations, we found that specific co-mutation patterns, such as those involving *TGFBR2*, *ARID1A*, and *PIK3CA*, are enriched and may drive aggressive tumor behavior. Co-mutation burden was highly correlated with TMB. It was also predictive of MSI status. These findings suggest that co-mutation analysis could serve as a proxy for genomic instability. This approach may help identify patients who are likely to benefit from immunotherapy[29,30].

Actionable alteration interactions differed between CHOL and GBC: *KRAS* mutations were subject to negative selection in CHOL, whereas *PIK3CA* mutations in GBC co-occurred with other targetable lesions. This implies that *KRAS*-mutant CHOL may be manageable with *KRAS*-targeted therapy alone, while *PIK3CA*-mutant GBC may

Table 1. Actionable alterations among subtypes.

Gene	Genotype	Subtype			*p*-Value
		WT (%)	SH (%)	MH (%)	
ATM	WT	1,650 (96.7)	399 (95.2)	183 (72.6)	< 0.001
	Mut	57 (3.3)	20 (4.8)	69 (27.4)	
BRAF	WT	1,659 (97.2)	401 (95.7)	240 (95.2)	0.117
	Mut	48 (2.8)	18 (4.3)	12 (4.8)	
*BRCA1**	WT	1,698 (99.5)	414 (98.8)	243 (96.4)	< 0.001
	Mut	9 (0.5)	5 (1.2)	9 (3.6)	
BRCA2	WT	1,663 (97.4)	399 (95.2)	232 (92.1)	< 0.001
	Mut	44 (2.6)	20 (4.8)	20 (7.9)	
ERBB2	Norm	1,594 (93.4)	406 (96.9)	231 (91.7)	0.009
	AMPL	113 (6.6)	13 (3.1)	21 (8.3)	
ERBB2	WT	1,636 (95.8)	405 (96.7)	223 (88.5)	< 0.001
	Mut	71 (4.2)	14 (3.3)	29 (11.5)	
*FGFR1**	WT	1,699 (99.5)	415 (99.0)	240 (95.2)	< 0.001
	Mut	8 (0.5)	4 (1.0)	12 (4.8)	
*FGFR2**	WT	1,684 (98.7)	414 (98.8)	241 (95.6)	0.006
	Mut	23 (1.3)	5 (1.2)	11 (4.4)	
*FGFR2**	Norm	1,699 (99.5)	416 (99.3)	252 (100.0)	0.411
	AMPL	8 (0.5)	3 (0.7)	0 (0.0)	
FGFR2	Norm	1,652 (96.8)	413 (98.6)	249 (98.8)	0.038
	FS/RA	55 (3.2)	6 (1.4)	3 (1.2)	
IDH1	WT	1,630 (95.5)	390 (93.1)	246 (97.6)	0.021
	Mut	77 (4.5)	29 (6.9)	6 (2.4)	
*IDH2**	WT	1,684 (98.7)	416 (99.3)	248 (98.4)	0.572
	Mut	23 (1.3)	3 (0.7)	4 (1.6)	
KRAS	WT	1,182 (69.2)	315 (75.2)	204 (81.0)	< 0.001
	Mut	525 (30.8)	104 (24.8)	48 (19.0)	
*MAP2K1**	WT	1,695 (99.3)	415 (99.0)	244 (96.8)	0.004
	Mut	12 (0.7)	4 (1.0)	8 (3.2)	
MDM2	Norm	1,597 (93.6)	400 (95.5)	248 (98.4)	0.004
	AMPL	110 (6.4)	19 (4.5)	4 (1.6)	
*MET**	WT	1,690 (99.0)	413 (98.6)	246 (97.6)	0.154
	Mut	17 (1.0)	6 (1.4)	6 (2.4)	
MET	Norm	1,671 (97.9)	409 (97.6)	247 (98.0)	0.924
	AMPL	36 (2.1)	10 (2.4)	5 (2.0)	
NF1	WT	1,671 (97.9)	407 (97.1)	156 (61.9)	< 0.001
	Mut	36 (2.1)	12 (2.9)	96 (38.1)	
NRAS	WT	1,670 (97.8)	409 (97.6)	247 (98.0)	0.937
	Mut	37 (2.2)	10 (2.4)	5 (2.0)	
PIK3CA	WT	1,601 (93.8)	412 (98.3)	181 (71.8)	< 0.001
	Mut	106 (6.2)	7 (1.7)	71 (28.2)	

*Fisher's exact test. WT: wildtype; Mut: mutation; SH: single-hit; MH: Multi-hit; AMPL: amplification; Norm: normal.

require combination targeted inhibition. Moreover, *ERBB2*- and *FGFR2*-mutant tumors harbored more complex co-mutational backgrounds than amplified/rearranged cases, possibly explaining the attenuated efficacy of targeted therapies[31,32]. This underscores the need for comprehensive genomic profiling beyond single-gene testing.

The adverse effect of DCMs on ICT outcomes was independent of clinicopathological variables, reinforcing the prognostic value of molecular stratification. Certain co-mutations were associated with poor prognosis. Interestingly, neither single gene mutation alone was significant. This suggests a synergistic biological effect between the genes. Similar phenomena have been observed in other malignancies, such as NSCLC[33] and colorectal cancer[14]. For instance, *KRAS/STK11* co-mutations in NSCLC define an immunotherapy-resistant subtype[12], analogous to our MH subtype in BTCs.

The proposed classification is both prognostic and biologically informative. The MH subtype had a high frequency of MSI. Despite this, tumors of this subtype derived only limited benefit from ICT. This suggests that deleterious mutations in these tumors may override the immunogenicity typically associated with MSI[34]. This has important clinical implications, as MSI status alone may be insufficient to guide immunotherapy in BTCs.

Enrichment analysis linked the MH subtype to pathways involved in drug resistance, immune evasion, and proliferation, providing mechanistic insights into its poor prognosis. The high frequency of actionable mutations in MH tumors (e.g., in *NF1*, *PIK3CA*, *ATM*, *ERBB2*, and *BRCA1/2*) suggests opportunities for targeted combination therapies[35,36]. For example, PARP inhibitors may be explored in *BRCA*-mutant cases[37], while *MEK* or *PI3K* inhibitors could be considered for tumors with *NF1* or *PIK3CA* mutations[38,39].

Mutational signature analysis further distinguished the three subtypes. The MH subtype was predominantly characterized by two specific signatures: DNA mismatch repair deficiency, and APOBEC activity. These signatures have been linked to hypermutation and immunotherapy response in other cancers[40,41]. However, in the MH subtype of BTCs, these features were associated with poor outcomes, underscoring the unique molecular context of this disease.

Several limitations should be noted. The ICT cohort was relatively small, and validation in larger prospective cohorts is needed. The biological mechanisms through which co-mutations influence the tumor microenvironment and treatment response remain to be elucidated. Functional studies and multi-omics approaches are required to uncover underlying pathways.

Conclusions

In summary, we have developed a practical and biologically relevant genomic classification system for BTCs based on co-mutation and single-mutation profiles. This framework may help guide patient selection for immunotherapy and identify new therapeutic targets for poor-prognosis subtypes. Future studies should validate this classification and explore subtype-specific treatment strategies.

Ethical statements

The study was conducted in accordance with the Declaration of Helsinki, and all procedures were approved by the Institutional Review Board of the First Affiliated Hospital of Nanjing Medical University.

Author contributions

The authors confirm their contribution to the paper as follows: study conception and design: Zhu W, Li X, Chen X, Wang D; data collection: Chen S, Wang F, Wang D; analysis and interpretation of results: Zhu W, Li X, Chen X, Wang D; draft manuscript preparation: Zhu W, Chen X, Wang D; All authors reviewed the results and approved the final version of the manuscript.

Data availability

Genome data is available in the Genome Sequence Archive for Humans (https://ngdc.cncb.ac.cn/gsa-human) under the HRA008984 dataset and the PRJCA031321 project.

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Conflict of interest

The authors declare that they have no conflict of interest.

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