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Original research

Genomic pathway alterations and their prognostic impact in biliary tract cancer: Insights from a multinational cohort treated with cisplatin, gemcitabine, and durvalumab

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A R T I C L E I N F O

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TMB
Prognostic biomarkers

A B S T R A C T

Background: Biliary tract cancers (BTC) are aggressive malignancies with limited therapeutic options. Recent advances have integrated immunotherapy into the standard of care, yet outcomes remain heterogeneous, emphasizing the need for molecular biomarkers to guide patient selection. This study aimed to assess the prognostic impact of pathway-level genomic alterations in patients with BTC treated with first-line cisplatin, gemcitabine, and durvalumab.

Methods: This retrospective, multicenter study included 735 patients with advanced BTC, of whom 197 underwent comprehensive genomic profiling using the FoundationOne® CDx assay. Pathways were classified based on the presence of key gene alterations, and their association with overall survival (OS) and progression-free survival (PFS) was assessed through univariate and multivariate analyses. Tumor mutational burden (TMB) was also evaluated as a potential biomarker.

Results: HRD/BRCAness pathway alterations were associated with significantly improved OS (23.3 vs. 13.8 months, HR 0.51, 95 % CI 0.27–0.93, $p = 0.0295$) and PFS (13.2 vs. 8.1 months, HR 0.53, 95 % CI 0.32–0.89, $p = 0.0153$). TGF-β pathway alterations were linked to longer PFS (16.0 vs. 8.1 months, HR 0.53, 95 % CI 0.28–0.99, $p = 0.0473$) but did not independently predict OS. High TMB (>10 mut/Mb) was associated with improved OS (NR vs. 11.0 months, HR 0.34, 95 % CI 0.14–0.85, $p = 0.0206$) and PFS (NR vs. 7.6 months, HR 0.40, 95 % CI 0.13–0.96, $p = 0.043$). However, no single pathway was significantly correlated with early treatment response.

*Conclusions: HRD/*BRCA*ness* and TGF-β pathway alterations, along with high TMB, emerged as potential prognostic biomarkers in patients with BTC treated with chemo-immunotherapy. These findings, if prospectively confirmed and validated, support the integration of molecular profiling into routine clinical practice to improve patient stratification and treatment outcomes in this challenging tumor type.

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1. Introduction

Biliary tract cancers (BTC) are a heterogenous group of malignancies with poor prognosis and limited response to systemic treatments. BTC are classified according to the anatomical tumor site in intrahepatic (iCCA), perihilar, and distal cholangiocarcinoma (eCCA), as well as gallbladder carcinoma (GBC). Over the past decades, BTC-related mortality has risen, as reported by the World Health Organization, underscoring the urgent need for improved treatment strategies [1–7].

Historically, systemic chemotherapy with gemcitabine and cisplatin has been the cornerstone of first-line treatment in advanced BTC [8]. The phase 3 TOPAZ-1 trial has led to a paradigm shift by demonstrating that the addition of durvalumab, a PD-L1 inhibitor, to gemcitabine and cisplatin significantly improves both overall survival (OS) and progression-free survival (PFS), establishing chemo-immunotherapy as the new standard of care [9]. The efficacy of this regimen has been further supported by updated analyses and real-world studies [10–13]. The phase 3 KEYNOTE-966 trial reinforced the efficacy of immunotherapy in increasing OS adding pembrolizumab to chemotherapy [14]. However, responses to these regimens are heterogeneous, and a substantial proportion of patients still experience early disease progression. This clinical variability highlights the urgent need for molecular biomarkers that can guide personalized treatment strategies and identify patients more likely to benefit from chemo-immunotherapy.

BTC are characterized by a complex genomic landscape, with alterations across a broad spectrum of signaling pathways, including DNA damage response and repair, receptor tyrosine kinases RAS/MAPK, PI3K/AKT/mTOR, TGF-β, MYC, NOTCH, and cell cycle regulators. Many of these pathways are biologically interconnected and may exert synergistic or antagonistic effects on tumor behavior and therapeutic response [15]. While previous studies have investigated the frequency of gene-level alterations in BTC, limited data exist on the prognostic impact of pathway-level alterations.

A real-world analysis of cisplatin, gemcitabine, and durvalumab identified three molecular clusters linked to overall response rate (ORR): Cluster 1 (31 % ORR) had chromatin modification mutations in all patients. Cluster 2 (50 % ORR) showed alterations in multiple pathways, including DNA repair, RTK/RAS, and TP53. Cluster 3 (0 % ORR) was defined by RTK/RAS and cell cycle apoptosis alterations [16].

Tumor mutational burden (TMB), an emerging biomarker of immunogenicity that measures non-synonymous alterations per megabase (Mb) by leveraging dedicated bioinformatics pipelines, has also gained attention in BTC [17,18]. Although TMB is generally lower in BTC compared to other solid tumors, high TMB may identify a subset of patients with increased neoantigen load and higher sensitivity to immune checkpoint inhibition. Understanding the relationship between TMB and specific oncogenic pathways could further refine patient stratification and improve the predictive accuracy of molecular profiling.

In this study, we retrospectively analyzed genomic data from a multicenter cohort of patients with BTC treated with first-line cisplatin, gemcitabine and durvalumab. Our aim was to comprehensively investigate the molecular landscape of BTC, identifying co-occuring pathways that may have a significant impact on clinical outcomes in terms of OS and PFS. Additionally, we explored the relationship between oncogenic pathways and TMB, aiming to uncover potential predictive biomarkers for treatment response.

2. Matherials and methods

2.1. Study population

The study population consisted of patients with unresectable, locally advanced, or metastatic BTC who received treatment at 39 clinical institutions across 11 countries (Italy, Germany, Austria, Spain, Portugal, United Kingdom, United States of America, Republic of Korea, China, Hong Kong Special Administrative Region of China, and Japan) from July 2021 to December 2023.

Data were retrospectively extracted from the reports of genomic analysis, performed by outsourced testing panel (FoundationOne CDx - F1CDx), able to analyze clinically relevant alterations across 324 cancer related genes integrating microsatellite instability (MSI-H) and TMB evaluation [19]. Specifically, pathways were classified according to the presence of their respective genes. Each pathway was assigned based on the identification of at least one pathogenic/likely pathogenic alteration in the referenced genes.

The classification allowed for a structured analysis of the genetic involvement across different pathways (Supp table 2). Detected alterations were categorized as either point mutations, fusions, rearrangements, or copy number variations, provided they were supported by validated amino acid substitutions, fusion partners, or quantitative scoring. Complex biomarkers such as MSI-H, TMB, and homologous recombination deficiency (HRD) were analyzed as separate variables. Each genomic alteration was subsequently assigned to one or more oncogenic pathways (e.g., RTK/RAS, PI3K, cell cycle, DNA damage response, WNT, NOTCH, among others) based on curated gene-function relationships.

Patients included in the analysis were treated with first line cisplatin (25 mg/m2, day 1 and 8), gemcitabine (1000 mg/m2, day 1 and 8), and durvalumab (1500 mg, day 1), administered intravenously on a 21-day cycle for up to eight chemotherapy cycles, followed by durvalumab monotherapy every 4 weeks until disease progression or unacceptable toxicity.

The study received approval from San Raffaele ethics Committees (number 113/INT/2021) and the local Ethics Committees at each participating center and adhered to Good Clinical Practice guidelines, the Declaration of Helsinki, local laws, and the EU General Data Protection Regulation (GDPR) 2016/679.

2.2. Statistical analysis

The primary endpoint of the study was the survival impact in terms of OS and PFS of the most common pathways (>3 % of patients) in a cohort of patients who received cisplatin, gemcitabine and durvalumab as first-line therapy. The secondary endpoints included the impact in terms of ORR and disease control rate (DCR) of the most common pathways (>3 % of patients).

Pathways were categorized as ‘most common’ when altered in at least 3 % of patients in the cohort. The 3 % threshold was chosen a priori as a pragmatic cut-off to balance inclusivity and statistical power: alterations below this frequency corresponded to fewer than 6 patients, which would preclude meaningful survival analyses and inflate the risk of spurious associations.Univariate survival analyses were performed using the Kaplan–Meier method, with comparisons assessed by the log-rank test. Variables associated with OS or PFS at a significance level of $p < 0.20$ in univariate analysis were entered into a multivariate Cox proportional hazards regression model. Hazard ratios (HR) and 95 % confidence intervals (CI) were calculated for each variable. For categorical variables, chi-square or Fisher’s exact tests were applied as appropriate. All p-values were two-sided, and a false discovery rate (FDR) correction was applied to account for multiple testing.

OS was defined as the time from treatment initiation to death, while PFS was the time from treatment initiation to disease progression, death, or last follow-up, whichever occurred first. ORR was assessed by the investigator as the proportion of patients achieving complete response (CR) or partial response (PR). DCR was the proportion of patients achieving ORR or stable disease (SD). Treatment responses were evaluated using computed tomography and categorized as CR, PR, SD, or progressive disease (PD) according to RECIST 1.1.

A comprehensive analysis of genomic alterations, co-occuring mutations across multiple signaling pathways, and complex genomic signatures (TMB) was performed. Odds ratios (ORs) were calculated to

assess the likelihood of co-occurrence between genetic alterations, with statistical significance evaluated using p-values and adjusted p-values ($p < 0.05$).

To assess the association between TMB and each pathway, Pearson's correlation coefficient was calculated, along with the p-value to determine statistical significance. TMB was treated as a continuous variable. The analysis was visualized using a scatter plot, where the Pearson correlation coefficient was plotted on the X-axis and the p-value on the Y-axis. Pathways with statistically significant associations were labeled in the plot for improved readability.

Statistical analysis was performed using MedCalc software (MedCalc® version 20.2).

3. Results

3.1. Molecular landscape and pathway interactions

Overall, the initial patient cohort comprised 735 individuals with advanced BTC treated with cisplatin and gemcitabine plus durvalumab; of these, 197 (26.8 %) underwent gene alteration analysis on tumor tissue samples with FoundationOne®CDx. Patients' characteristics are presented in Table 1 and 2 shows the percentage of altered pathways.

To minimize the risk of selection bias, we compared baseline clinical characteristics of patients who underwent genomic profiling (n = 197) with those who did not (n = 538). No clinically relevant differences were observed between the two groups in terms of age, sex, ECOG performance status, tumor site, or disease stage (Supplementary Table 1), supporting the representativeness of the tested cohort.

The binary heatmap represents the activation status of multiple biological pathways across 197 samples (Fig. 1).

We analyzed the distribution of key oncogenic pathway mutations across different anatomical sites of BTC, including iCCA, eCCA, and GBC (Fig. 2). The heatmap illustrates distinct mutational landscapes across these tumor sites, with RTK/RAS and TGF-β pathways showing statistically significant differences. The RTK/RAS pathway had the highest mutation rate in iCCA (46.2 %), compared to 37.7 % in GBC and only 12.5 % in eCCA, with a significant difference across locations ($p = 0.006$). The TGF-β pathway also displayed a notable variation, with 13.5 % of iCCA cases harboring mutations, compared to 3.3 % in GBC and 0 % in eCCA ($p = 0.008$).

The analysis identified significant co-occurrences among key oncogenic pathways (Fig. 3). Notably, the DNA Damage Control pathway exhibited a strong co-occurrence with the *HRD*/BRCA*ness* pathway (OR = 20.28), suggesting a potential synergy between these alterations. Additionally, FGF/FGFR alterations were significantly associated with the PI3K pathway (OR = 5.90), reinforcing the role of fibroblast growth factor signaling in oncogenesis.

Adjusted p-values confirmed the robustness of these associations, with the *HRD*/BRCA*ness* -DNA Damage Control co-occurrence remaining highly significant (adjusted $p < 0.0001$). Similarly, the association between FGF/FGFR and PI3K retained significance (adjusted $p = 0.0198$).

The analysis revealed strong interconnections between the MAPK and MYC pathways (OR = 3.18, $p = 0.14$), though statistical significance was not reached after multiple comparison adjustments. Conversely, the NOTCH pathway displayed a significant association with the Cyclin pathway (OR = 9.26, adjusted $p = 0.0024$), indicating potential cooperative effects in tumorigenesis.

3.2. Prognostic impact of pathway alterations

At univariate analysis for OS *HRD*/BRCA*ness* alteration was associated with longer median OS (23.3 vs. 13.8 months, HR 0.51; 95 % CI 0.28–0.93; $p = 0.0295$) compared to that in patients without *HRD*/BRCA*ness* alteration (Fig. 4A). No other pathways were associated with a different outcome, and the HRs for all pathways are summarized in the forest plot (Fig. 5A). After adjustment for variables with a prognostic impact in the univariate analysis (including factors with $p < 0.2$), the multivariate analysis for OS confirmed the positive prognostic role of *HRD*/BRCA*ness* alteration (Table 3).

At univariate analysis for PFS *HRD*/BRCA*ness* (Fig. 4B) and TGF-beta (Fig. 4C) alterations were associated with longer median PFS (13.2 vs. 8.1 months, HR 0.53; 95 % CI 0.32–0.89; $p = 0.0153$ and 16.0 vs. 8.1 months, HR 0.53; 95 % CI 0.28–0.99; $p = 0.0473$) compared to that of patients without *HRD/BRCAness* alteration and TGF-beta alteration, respectively. No other pathways were associated with a different outcome, and the HRs for all pathways are summarized in the forest plot (Fig. 5B). After adjustment for variables with a prognostic impact in the univariate analysis (including factors with $p < 0.2$), the multivariate analysis for PFS confirmed the positive prognostic role of *HRD*/BRCA*ness* alteration but not of TGF-beta (Table 3).

No pathways correlated with a higher ORR or DCR (Table 4 and Fig. 6).

When stratifying by primary tumor site, *HRD*/BRCA*ness* pathway alterations were associated with a clear survival advantage in iCCA (HR 0.63, 95 % CI 0.44–0.93; $p = 0.041$). Similarly, alterations in the TGF-β pathway correlated with improved outcomes in intrahepatic tumors (HR 0.71, 95 % CI 0.52–0.95; $p = 0.045$). In contrast, no consistent favorable associations were observed in eCCA or GBC.

Only three patients received targeted therapies based on the identified genomic alteration; therefore, the small sample size does not allow for further statistical analyses.

3.3. Pathway correlations with TMB dynamics and outcome

We analyzed TMB values from 48 patients (24.4 %). The median TMB was 3.78 (range 0.00–50.43), with a mean of 5.45 (SD 8.07). The analysis identified three pathways with significant positive correlations with TMB (Fig. 7). The *HRD*/BRCA*ness* pathway showed a strong positive correlation ($r = 0.526$, $p = 0.0001$). Similarly, the DNA Damage Control pathway demonstrated a significant positive correlation with TMB ($r = 0.302$, $p = 0.037$), indicating its potential role in increased mutational burden. Moreover, the TGF-beta pathway exhibited the strongest positive correlation with TMB ($r = 0.669$, $p < 0.0001$), reinforcing its potential involvement in mutational dynamics. No other pathways showed statistically significant correlations with TMB. The scatter plot illustrates the distribution of correlations, with a significance threshold ($p = 0.05$) marked to distinguish relevant associations from non-significant ones.

At univariate analysis for OS TMB was associated with longer median OS (Not Reach vs. 11.0 months, HR 0.34; 95 % CI 0.14–0.85; $p = 0.0206$) compared patients with TMB< 10 (Fig. 8A).

At univariate analysis for PFS TMB ≥ 10 was associated with longer median PFS (Not Reach vs. 7.6 months, HR 0.40; 95 % CI 0.13–0.96; $p = 0.043$) compared patients with TMB< 10 (Fig. 8B).

TMB> 10 did not correlate with a higher ORR or DCR (Supp Fig. 1). The correlation between TMB as a continue variable and ORR was weakly positive ($r = 0.20$, $p = 0.196$) (Supp Fig. 2A). Similarly, the correlation between TMB as a continue variable and DCR was weak ($r = 0.10$, $p = 0.515$), also lacking statistical significance (Supp Fig. 2B). The scatter plots with regression lines illustrate these trends, with no clear relationship between increasing TMB and improved ORR or DCR.

4. Discussion

In this large, real-world, multicenter study, we provide a comprehensive genomic analysis of patients with advanced BTC treated with first-line cisplatin, gemcitabine, and durvalumab.

The most frequently altered pathways in our cohort, including RTK/RAS (35.9 %), DNA damage control (25.7 %), chromatin remodeling (23.3 %), cell cycle/apoptosis (22.3 %), and TP53 (21.8 %), reflect the known molecular heterogeneity of BTC and are consistent with previous

Table 1
Baseline characteristics of patients and frequency of mutated pathways.

Pathway	Mutation %																	
	Cell Cycle Apoptosis	Chromatin Modification	Cyclin	DNA Damage Control	FGF/ FGFR	HH	HIPPO	HRD/ BRACness	MAPK	MYC	NOTCH	NRF2	PI3K	RTK RAS	STAT	TGF - b	TP53	Transcriptional Regulation	WNT
Group																			
Alkaline phosphatase normal value	30.6	25.8	24.2	25.8	6.5	0.0	0.0	17.7	1.6	4.8	3.2	0.0	27.4	37.1	3.2	3.2	25.8	6.5	8.1
Alkaline phosphatase no normal value	17.1	17.1	17.1	21.4	7.1	0.0	1.4	10.0	2.9	2.9	4.3	1.4	17.1	34.3	2.9	5.7	20.0	2.9	5.7
Gamma glutamyl transferase normal value	24.2	25.0	21.9	25.0	9.4	0.8	0.8	15.6	1.6	3.9	4.7	0.8	25.8	38.3	3.1	3.9	21.1	6.3	8.6
Gamma glutamyl transferase no normal value	14.0	18.0	12.0	22.0	2.0	0.0	0.0	8.0	2.0	0.0	4.0	0.0	14.0	24.0	4.0	10.0	20.0	2.0	2.0
NLR < 3	28.7	24.1	24.1	29.9	5.7	1.1	0.0	14.9	1.1	3.4	3.4	1.1	21.8	36.8	1.1	8.0	26.4	5.7	10.3
NLR≥ 3	16.8	21.2	15.0	22.1	9.7	0.0	0.9	14.2	1.8	3.5	5.3	0.9	21.2	35.4	4.4	3.5	18.6	4.4	4.4
Male	17.7	20.8	20.8	19.8	8.3	0.0	0.0	14.6	3.1	2.1	3.1	2.1	20.8	34.4	2.1	4.2	16.7	4.2	5.2
Female	26.4	25.5	19.1	30.9	7.3	0.9	0.9	14.5	0.0	4.5	5.5	0.0	20.9	37.3	3.6	6.4	26.4	5.5	8.2
Age ≤ 70	23.6	20.8	16.7	29.2	4.2	0.0	0.0	16.7	1.4	2.8	4.2	1.4	12.5	34.7	2.8	4.2	26.4	8.3	4.2
Age > 70	19.2	24.8	20.0	21.6	9.6	0.8	0.8	13.6	1.6	4.0	4.0	0.8	25.6	35.2	3.2	6.4	16.8	2.4	7.2
ALT no normal value	23.8	21.4	16.7	21.4	9.5	0.0	0.0	11.9	0.0	4.8	0.0	2.4	23.8	42.9	2.4	4.8	19.0	7.1	9.5
ALT normal value	21.5	23.4	19.6	26.6	7.6	0.6	0.6	15.2	1.9	3.2	5.7	0.6	20.3	34.2	3.2	5.7	22.8	4.4	6.3
AST no normal value	20.3	24.3	13.5	20.3	8.1	0.0	0.0	12.2	0.0	1.4	1.4	0.0	14.9	33.8	4.1	4.1	17.6	4.1	5.4
AST normal value	24.1	19.8	19.0	29.3	8.6	0.9	0.0	14.7	0.9	3.4	5.2	1.7	22.4	36.2	1.7	5.2	26.7	5.2	5.2
Bilirubin no normal value	25.0	23.2	18.9	27.4	7.9	0.6	0.6	15.2	1.2	3.7	5.5	0.6	20.7	35.4	3.7	5.5	24.4	4.9	6.7
Bilirubin normal value	9.1	18.2	21.2	9.1	9.1	0.0	0.0	3.0	3.0	3.0	0.0	3.0	27.3	36.4	0.0	6.1	6.1	6.1	9.1
Ca19.9 normal value	20.5	18.9	18.2	23.5	6.8	0.0	0.0	15.2	0.8	3.0	5.3	0.8	18.9	35.6	2.3	2.3	20.5	6.1	5.3
Ca19.9 no normal value	24.6	29.2	20.0	29.2	7.7	1.5	1.5	13.8	3.1	4.6	1.5	0.0	24.6	33.8	4.6	10.8	24.6	1.5	10.8
CEA no normal value	21.9	26.3	14.9	27.2	7.9	0.9	0.9	16.7	1.8	3.5	2.6	0.0	20.2	35.1	2.6	6.1	23.7	1.8	7.9
CEA normal value	22.5	17.5	25.0	22.5	6.3	0.0	0.0	12.5	1.3	3.8	6.3	1.3	22.5	33.8	2.5	2.5	18.8	8.8	6.3
ECOG PS = 0	23.8	23.8	19.8	24.6	8.7	0.8	0.8	14.3	0.8	3.2	2.4	1.6	22.2	31.0	3.2	5.6	20.6	2.4	7.1
ECOG PS > 0	20.0	22.5	20.0	27.5	6.3	0.0	0.0	15.0	2.5	3.8	7.5	0.0	18.8	43.8	2.5	5.0	23.8	8.8	6.3
Locally advanced	23.3	23.3	22.7	25.8	8.0	0.6	0.6	12.3	1.8	3.1	4.3	1.2	22.7	38.0	3.7	6.1	20.9	4.9	8.0
Metastatic	18.6	23.3	9.3	25.6	7.0	0.0	0.0	23.3	0.0	4.7	4.7	0.0	14.0	27.9	0.0	2.3	25.6	4.7	2.3

Table 2
Distribution of genomic pathway alterations in the study population.

	Percentage
RTK RAS	35.9
DNA Damage Control	25.7
Chromatin Modification	23.3
Cell Cycle Apoptosis	22.3
TP53	21.8
PI3K	20.9
Cyclin	19.9
HRD/BRACness	14.6
FGF/FGFR	7.8
WNT	6.8
TGF-b	5.3
Transcriptional Regulation	4.9
NOTCH	4.4
MYC	3.4
STAT	3.4
MAPK	1.5
NRF2	1.0
HH	0.5
HIPPO	0.5

reports [5]. Subtype-specific differences were also observed, such as the enrichment of RTK/RAS alterations in iCCA (46.2 %) and GBC (37.7 %), and PI3K pathway mutations in eCCA (21.9 %). TGF-β pathway alterations were enriched in iCCA (13.5 %), but rare in GBC (3.3 %) and eCCA (0), aligning with preclinical evidence linking this pathway to lymph node involvement, distant metastases, and tumor relapse. TGF-β pathway alterations within the tumor microenvironment has been associated with resistance to PD-L1 pathway inhibitors, further implicating this pathway in BTC progression [20–22]. Our analysis reveals significant associations between specific oncogenic pathway alterations and clinical outcomes, suggesting that genomic profiling may offer valuable prognostic insights in the era of chemo-immunotherapy for BTC. Alterations in the *HRD*/BRCA*ness* pathway were associated with improved OS and PFS, highlighting its potential as a positive prognostic marker.

In line with the well-recognized heterogeneity of BTC, our exploratory analyses stratified by anatomical site revealed that the prognostic impact of genomic pathways was not uniform across subgroups. Alterations in *HRD/BRCA-related* and TGF-β signaling pathways were associated with improved outcomes in iCCA, whereas no consistent favorable associations emerged in eCCA or GBC. This lack of signal in the latter subgroups is likely influenced not only by distinct biological features but also by the relatively small sample sizes, which limit statistical power. Overall, these findings underscore the importance of considering anatomical heterogeneity when interpreting molecular correlates in BTC and suggest that future studies should further validate these signals in larger, site-specific cohorts.

In contrast, the RTK/RAS pathway, frequently altered in iCCA, was not significantly associated with any clinical outcomes. Additionally, pathways involved in DNA damage response and repair, such as DNA Damage Control and TGF-β, also demonstrated significant associations with survival, further emphasizing the complex interplay between genomic instability and tumor progression in BTC. These pathways, although associated with improved OS and PFS, were not correlated with higher ORR or DCR, suggesting that their prognostic value may be more related to long-term disease control rather than initial tumor shrinkage or disease stabilization. It is possible that the benefits conferred by alterations in DNA repair-related pathways manifest over time through mechanisms such as enhanced sensitivity to platinum-based chemotherapy or immunogenic effects resulting from increased genomic instability [23–25]. Moreover, the lack of association with ORR and DCR underscores the limitations of traditional response criteria in capturing the full clinical benefit of chemo-immunotherapy in molecularly defined subgroups. Furthermore, we measured TMB in a subset of 48 patients (24.4 % of the cohort), revealing a median TMB of 3.78 mutations/Mb and a mean of 5.45, a value higher than that reported in previous studies [26–30]. Despite the limited sample size, this finding suggests potential variability in mutational landscapes across different patient populations. A high TMB (≥ 10 mut/Mb) has been associated with favorable responses to immune checkpoint inhibitors (ICIs) in several types of cancer, probably due to the generation of a substantial pool of immune neoantigenes, ultimately leading to heightened tumor immunogenicity [17,18,31].

In BTC, MSI-H status is known to co-occur with high TMB and PD-L1 positivity, both linked to improved PFS and OS in patients receiving immunotherapy [32].

Nevertheless, the prognostic and predictive role of TMB in BTC remains controversial. Unlike other tumor types where high TMB is a well-established predictor of immunotherapy benefit [33], its role in hepatobiliary cancers is still not clear. Several studies have reported low prevalence of high TMB (> 10 mut/Mb) and inconsistent associations with response to or survival on immunotherapy. While a few analyses

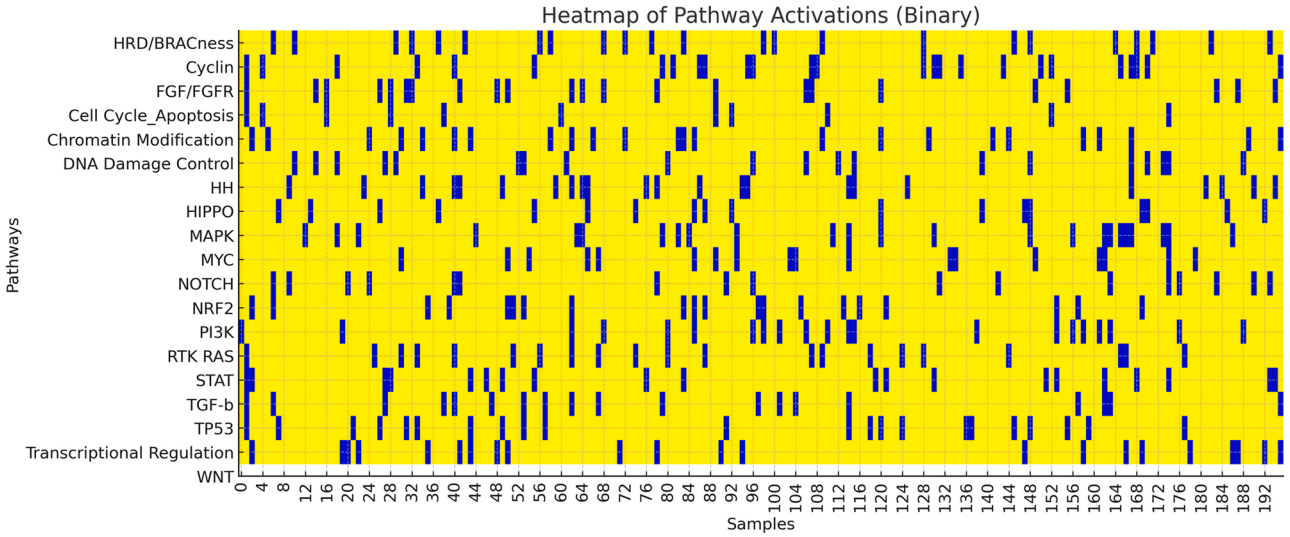

Fig. 1. Binary heatmap of pathway activation in BTC samples. Each column represents an individual sample, and each row corresponds to a specific oncogenic pathway. Yellow indicates no activation, while blue indicates pathway activation. Each pathway is represented as a row, and each sample as a column, with activation indicated by blue (active) and non-activation by yellow (inactive).

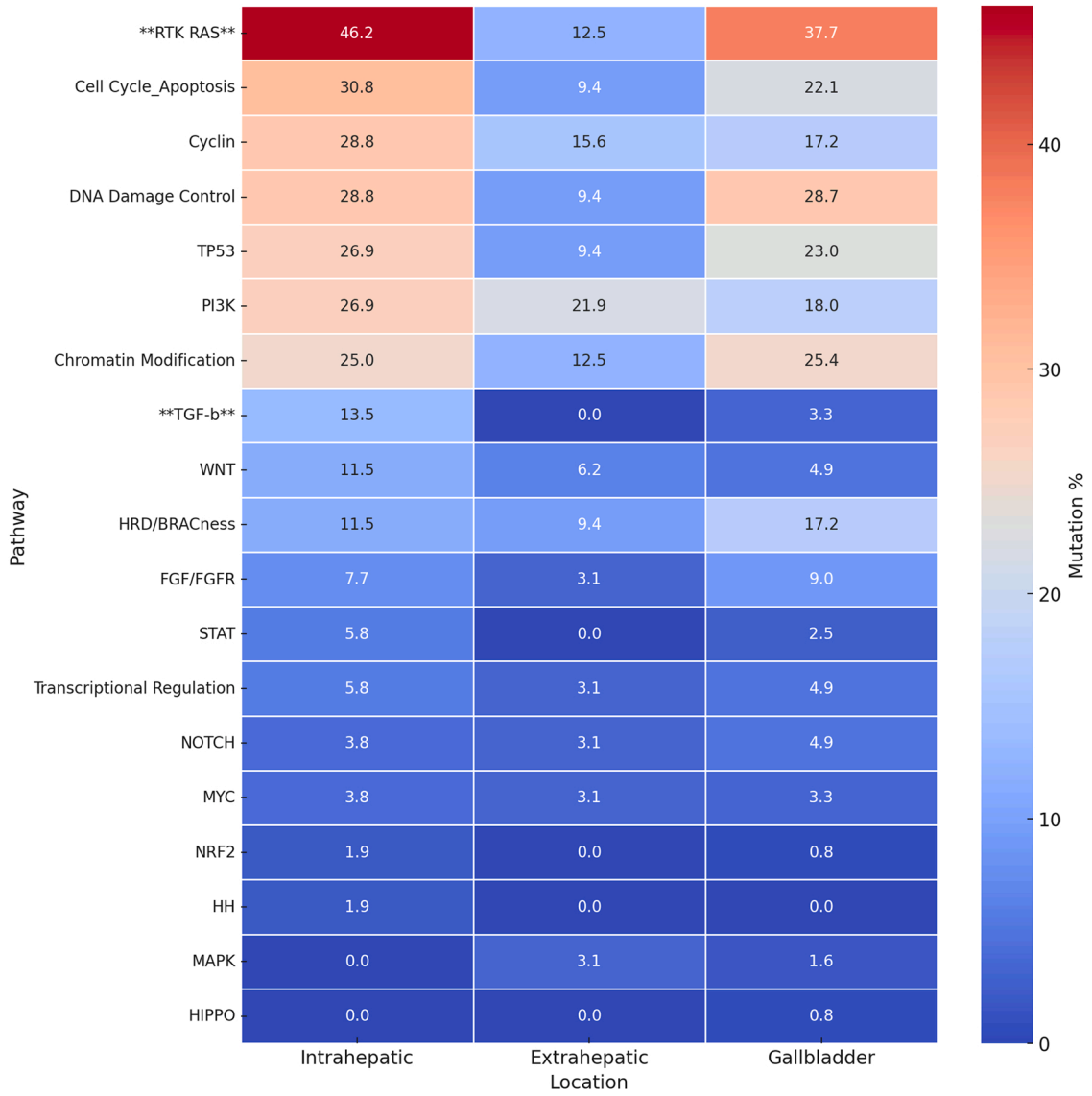

Fig. 2. Distribution of pathway alterations by anatomical location in BTC. The heatmap illustrates the frequency of pathway alterations across intrahepatic cholangiocarcinoma (iCCA), extrahepatic cholangiocarcinoma (eCCA), and gallbladder carcinoma (GBC). Pathways with significant differences in mutation rates are indicated (e.g., RTK/RAS, TGF-β).

suggest that high TMB may be linked to improved outcomes in selected cohorts, others have shown no significant correlation [34–37].

In our population, high TMB (>10 mut/Mb) was significantly associated with improved OS and PFS, but did not correlate with ORR or DCR. This weak correlation between TMB and ORR confirms that additional factors influence immunotherapy outcomes in BTC. The prognostic value of genomic alterations and TMB in BTC suggests that integrating molecular profiling into clinical decision-making could enable a more personalized therapeutic approach. Further studies are needed to assess whether these specific pathways contribute to a more immunogenic tumor profile in BTC.

Interestingly, three pathways, *HRD*/BRCA*ness*, DNA Damage Repair, and TGF-β signaling, demonstrated a positive correlation with TMB, reinforcing their role in promoting genomic instability and increasing mutational load. Previous studies have also associated *BRCA1/2* alterations with higher TMB and a greater prevalence of MSI-H in iCCA [27], further supporting the idea that deficiencies in homologous recombination can lead to a more immunogenic tumor phenotype.

Among the evaluated pathways, *HRD*/BRCA*ness* showed the strongest prognostic signal, consistent with evidence from other cancers. These alterations, linked to defective DNA repair and higher genomic instability, may enhance sensitivity to platinum-based chemotherapy and ICIs [23]. Their association with high TMB supports this hypothesis. *HRD*/BRCA*ness* may thus represent a relevant biomarker to guide chemo-immunotherapy and PARP inhibitor use in BTC. A recent phase II study results suggest encouraging efficacy for olaparib in patients with BTC and HRD/BRCAness mutations [38]. Future studies should explore PARP and ICI combinations in HRD-positive patients to expand therapeutic options. TGF-β pathway alterations were also associated with prolonged PFS, suggesting a potential role in modulating tumor progression. Mechanistically, this may reflect differential sensitivity to DNA-damaging agents or immune modulation via the tumor microenvironment. Although preclinical studies have implicated TGF-β signaling in immune evasion and resistance to PD-L1 blockade [21,22], its precise role in BTC warrants further investigation.

Although TMB did not predict early treatment response, its association with long-term outcomes and specific oncogenic pathways supports the development of refined, pathway-based biomarker strategies to optimize patient selection for immunotherapy. Future clinical trials should explore composite models integrating TMB with additional

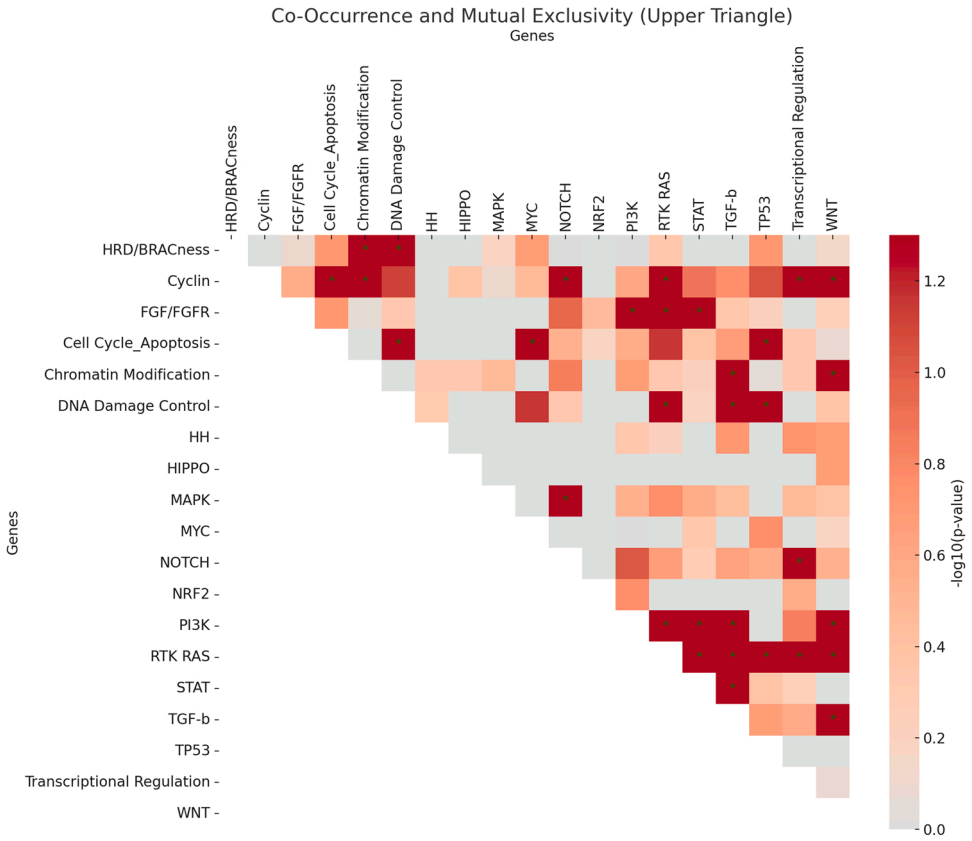

Fig. 3. Co-occurrence and mutual exclusivity of pathway alterations in BTC samples. The upper triangle heatmap shows significant co-occurrence (red) and mutual exclusivity (grey) of pathway alterations. Darker shades indicate stronger associations.

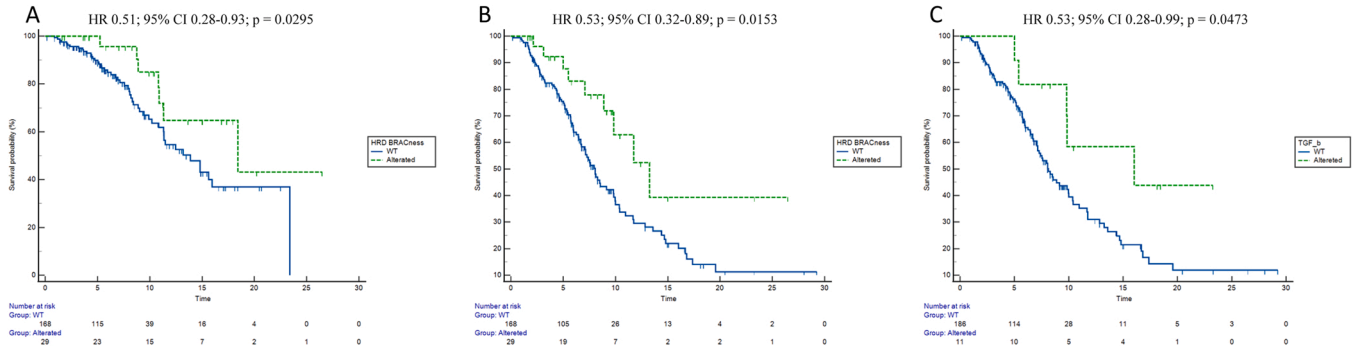

Fig. 4. Kaplan-Meier survival curves for overall survival (OS) and progression-free survival (PFS) by *HRD*/BRCA*ness* and TGF-β pathway status. (A) OS in *HRD*/BRCA*ness* altered vs. non-altered patients. (B) PFS in *HRD*/BRCA*ness* altered vs. non-altered patients. (C) PFS in TGF-β altered vs. non-altered patients.

molecular and immune parameters. Notably, no specific signaling pathways significantly correlated with ORR or DCR suggesting that genomic alterations alone may not fully predict treatment response in BTC.

The observed significant co-occurrences among key oncogenic pathways underscore the complex interplay underlying tumorigenesis. The strong association between the DNA Damage Control pathway and the *HRD*/BRCA*ness* pathway (OR = 20.28) highlights a potential synergy in supporting genomic instability and homologous recombination deficiency. The positive correlation between FGF/FGFR alterations and the PI3K pathway (OR = 5.90) suggests a mechanistic link whereby FGF signaling may contribute to activation of the PI3K/AKT pathway, promoting proliferative and survival signals. Additionally, the significant association between NOTCH signaling and the Cyclin pathway (OR = 9.26, adjusted p = 0.0024) indicates a coordinated regulation of cell cycle progression and differentiation processes, which may have implications for targeted therapeutic strategies. These findings support interactive oncogenic mechanisms in patients with BTC suggesting that co-occurrent alterations could influence tumor behavior and response to therapy, warranting further functional studies to dissect these interactions and their clinical relevance.

Despite promising advances in decodifying molecular landscape of patients with BTC, our study has several limitations. First, as a retrospective analysis, it is subject to selection bias and potential confounding factors that may affect the validity of the results. We exclusively used the FoundationOne® CDx assay for genomic profiling to minimize the technical variability associated to the implementation of different testing strategies While this approach aimed to ensure data consistency and comparability, it also limited the overall sample size, potentially reducing the statistical power of our analyses and restricting the

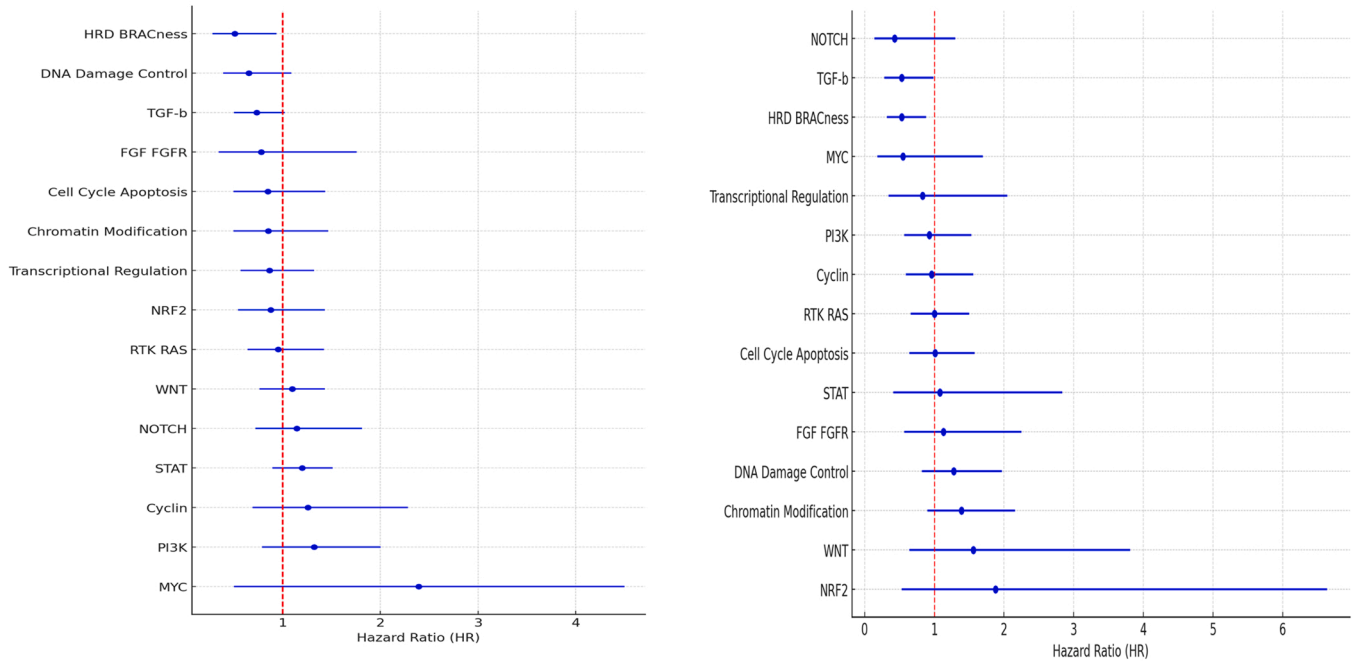

Fig. 5. Forest plot summarizing hazard ratios (HRs) for overall survival (OS) and progression-free survival (PFS) across different pathways. Only *HRD/*BRCA*ness* and TGF-β pathways demonstrated a statistically significant impact on survival.

identification of relevant variants not covered by this specific panel. Furthermore, the subset of patients with available genomic data remains relatively small compared to the overall population, which may reduce the statistical power of certain analyses.

Second, due to the real-world nature of the study, variability in treatment administration, tumor assessments, and follow-up protocols across participating institutions may have influenced clinical outcomes. The assessment of PFS is heterogeneous due to differences in radiologic evaluation schedules and clinical practice across centers, which may have introduced biases and affected the accuracy of PFS estimation.

Third, our analysis focused exclusively on pathogenic variants, excluding variants of uncertain significance, which may have affected the observed mutation detection rate of clinically relevant alterations. Consequently, the true prevalence of genomic alterations in this population may have been underestimated.

Finally, the genomic profiling platform used (FoundationOne® CDx) is affected by intrinsic limitations in successfully detecting large structural variants or low-frequency mutations, which could further impact the comprehensiveness of mutation landscape.

In conclusion, *HRD/*BRCA*ness* alterations emerged as the strongest genomic marker of improved survival in patients with BTC treated with cisplatin, gemcitabine, and durvalumab. Their prognostic value was confirmed in multivariate analysis and supported by a strong correlation with higher TMB, suggesting increased genomic instability and immunogenicity. TMB $\geq$ 10 mut/Mb was also associated with better OS and PFS, though not with response, reinforcing its role as a marker of durable benefit rather than radiologic shrinkage. However, given the limited number of patients with available TMB data, these findings should be interpreted as exploratory, and validation in larger, prospective cohorts will be essential to confirm the clinical utility of TMB in BTC. The co-occurrence of *HRD/*BRCA*ness* with DNA Damage Control further supports a distinct molecular profile linked to favorable outcomes. Taken together, these findings suggest that HRD status and TMB could help stratify patients and refine therapeutic strategies in BTC. Integrating molecular profiling into routine practice may improve prognostic assessment and support the development of personalized therapies.

CRediT authorship contribution statement

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Table 3
Univariate and multivariate analysis of overall survival (OS) and progression-free survival (PFS) by pathway alterations. This table shows the hazard ratios (HRs) and 95 % confidence intervals (CIs) for different pathways, highlighting those with significant associations with survival outcomes.

	Overall survival				Progression free survival			
	Univariate		Multivariate		Univariate		Multivariate	
Patients characteristcs	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
MYC	1	0,2741			1	0.3050		
WT	2.39 (1.50-4.50)				1.79 (0.59-5.49)			
Altereted								
PI3K	1	0,2785			1	0.7954		
WT	1.32 (0.78-2.00)				1.07 (0.65-1.74)			
Altereted								
Cyclin	1	0,4504			1	0.8757		
WT	1.25 (0.69-2.28)				1.04 (0.64-1.69)			
Altereted								
STAT	1	0,3987			1	0.9043		
WT	1.20 (0.89-1.51)				0.92 (0.35-2.42)			
Altereted								
NOTCH	1	0,4153			1	0.1394		
WT	1.14 (0.72-1.81)				2.28 (0.76-6.80)			
Altereted								
WNT	1	0,4321			1	0.3227		
WT	1.09 (0.76-1.43)				0.64 (0.26-1.55)			
Altereted								
RTK RAS	1	0,5023			1	0.9992		
WT	0.95 (0.64-1.42)				1.00 (0.67-1.50)			
Altereted								
NRF2	1	0,3121			1	0.3263		
WT	0.87 (0.54-1.43)				0.53 (0.15-1.87)			
Altereted								
Transcriptional Regulation	1	0,3492			1	0.7017		
WT	0.86 (0.56-1.32)				1.19 (0.49-2.92)			
Altereted								
Chromatin Modification	1	0,5643			1	0.1344		
WT	0.85 (0.49-1.46)				0.71 (0.46-1.11)			
Altereted								
Cell Cycle Apoptosis	1	0,5363			1	0.9565		
WT	0.84 (0.49-1.43)				0.99 (0.63-1.54)			
Altereted								
FGF FGFR	1	0,5474			1	0.7100		
WT	0.77 (0.34-1.75)				0.88 (0.44-1.74)			
Altereted								
TGF-b	1	0,2178			1	0.0473	1	0.2217
WT	0.73 (0.50-1.02)				0.53 (0.28-0.99)		0.55 (0.21-1.43)	
Altereted								
DNA Damage Control	1	0,103	1	0,243	1	0.2640		
WT	0.65 (0.38-1.09)		0.72 (0.41-1.18)		0.78 (0.51-1.20)			
Altereted								
HRD BRACness	1	0,0295	0.22 (0.07-0.66)	0.0066	1	0.0153	1	0.0043
WT	0.51 (0.27-0.93)				0.53 (0.32-0.89)		0.34 (0.16-0.71)	
Altereted								
Gender	1	0.68			1	0.3292		
Male	1.10 (0.69-1.78)				1.21 (0.82-1.80)			
Female								
Age	1	0.61			1	0.4297		
<70	0.87 (0.53-1.44)				0.85 (0.57-1.27)			
≥70								
Albumin	1	0.01	1	0.054	1	0.0052	1	0.1091
No NV	0.33 (0.14-0.80)		0.50 (0.25-1.01)		0.37 (1.19-0.74)		0.70 (0.46-1.08)	
NV								
ALT	1	0.004	1	0.56	1	0.0083	1	0.0298
No NV	0.39 (0.20-0.74)		0.77 (0.32-1.83)		0.48 (0.28-0.83)		0.56 (0.33-0.94)	
NV								
AST	1	0.02	1	0.37	1	0.0137	1	0.8893
No NV	0.55 (0.33-0.92)		0.71 (0.33-1.50)		0.59 (0.38-0.90)		1.03 (0.64-1.66)	
NV								
Bilirubin	1	0.39			1	0.0911		
No NV	0.74 (0.37-1.48)				0.60 (0.34-1.08)			
NV								
Ca19-9	1	0.03	1	0.92	1	0.0642		
No NV	0.58 (0.35-0.96)		0.96 (0.41-2.24)		0.67 (0.44-1.02)			
NV								
CEA	1	0.002	1	0.22	1	0.0661		
No NV	0.44 (0.26-0.75)		0.66 (0.33-1.29)		0.67 (0.42-1.03)			
NV								

(continued on next page)

Table 3 (*continued*)

	Overall survival				Progression free survival			
	Univariate		Multivariate		Univariate		Multivariate	
Patients characteristcs	**HR (95% CI)**	**P-value**	**HR (95% CI)**	**P-value**	**HR (95% CI)**	**P-value**	**HR (95% CI)**	**P-value**
Dreinage	1	0.06	1	0.17	1	0.3806		
Yes	0.55 (0.28-1.04)		0.47 (0.16-1.37)		0.80 (0.48-1.32)			
No								
ECOG	1	<0.0001	1	0.007	1	<0.0001	1	0.0002
>0	0.28 (0.16-0.49)		0.20 (0.13-0.68)		0.35 (0.23-0.54)		0.37 (0.22-0.62)	
0								
Location	1	0.051	1	0.56	1	0.04	1	0.0475
Extrahepatic	1.63 (0.76-3.52)		1.45 (0.66-1.68)		1.91 (0.99-3.69)		2.35 (1.09-5.07)	
Gallbladder	2.03 (1.22-3.40)		1.56 (0.89-2.45)		1.78 (1.15-2.74)		1.80 (1.01-3.23)	
Intrahepatic								
NLR	1	0.0003	1	0.051	1	0.0036	1	0.2411
<3	2.46 (1.51-4.01)		2.44 (0.99-5.98)		1.81 (1.21-2.69)		1.32 (0.82-2.13)	
≥3								
Surgery	1	0.04	1	0.17	1	0.1566		
No	0.61 (0.37-0.99)		0.54 (0.22-1.30)		0.74 (0.49-1.12)			
Yes								

Table 4
Tumor response according to genomic pathway status (mutated vs wild-type).

Pathway	ORR % (Altered)	ORR % (Not Altered)	P-value	DCR % (Altered)	DCR % (Not Altered)	P-value
HRD/BRCA*ness*	28.6	37.0	0.52	89.3	80.0	0.37
Cyclin	41.0	34.4	0.56	79.5	81.8	0.92
FGF/FGFR	56.3	33.9	0.13	87.5	80.8	0.75
Cell Cycle Apoptosis	39.1	34.7	0.71	76.1	83.0	0.41
Chromatin Modification	40.9	34.2	0.53	86.4	79.9	0.45
DNA Damage Control	35.3	35.9	1.00	84.3	80.3	0.67
MYC	28.6	36.0	1.00	71.4	81.7	0.85
NOTCH	22.2	36.4	0.61	55.6	82.6	0.11
PI3K	28.6	37.7	0.36	71.4	84.1	0.10
RTK RAS	33.8	36.9	0.78	78.9	82.8	0.63
TGF-b	36.4	35.7	1.00	90.9	80.8	0.66
TP53	36.4	35.6	1.00	81.8	81.2	1.00
Transcriptional Regulation	30.0	36.1	0.96	60.0	82.5	0.17
WNT	21.4	36.9	0.38	78.6	81.6	1.00

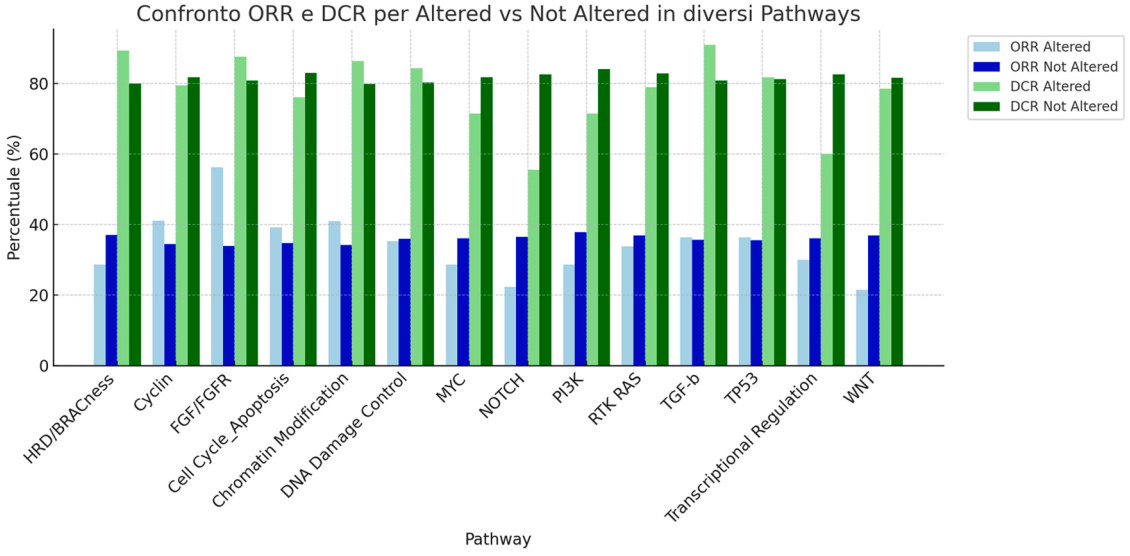

Fig. 6. Correlation of TMB with pathway alterations. The scatter plot illustrates the Pearson correlation coefficients and corresponding p-values for each pathway. Pathways with significant associations are labeled. The red dashed line indicates the significance threshold ($p = 0.05$).

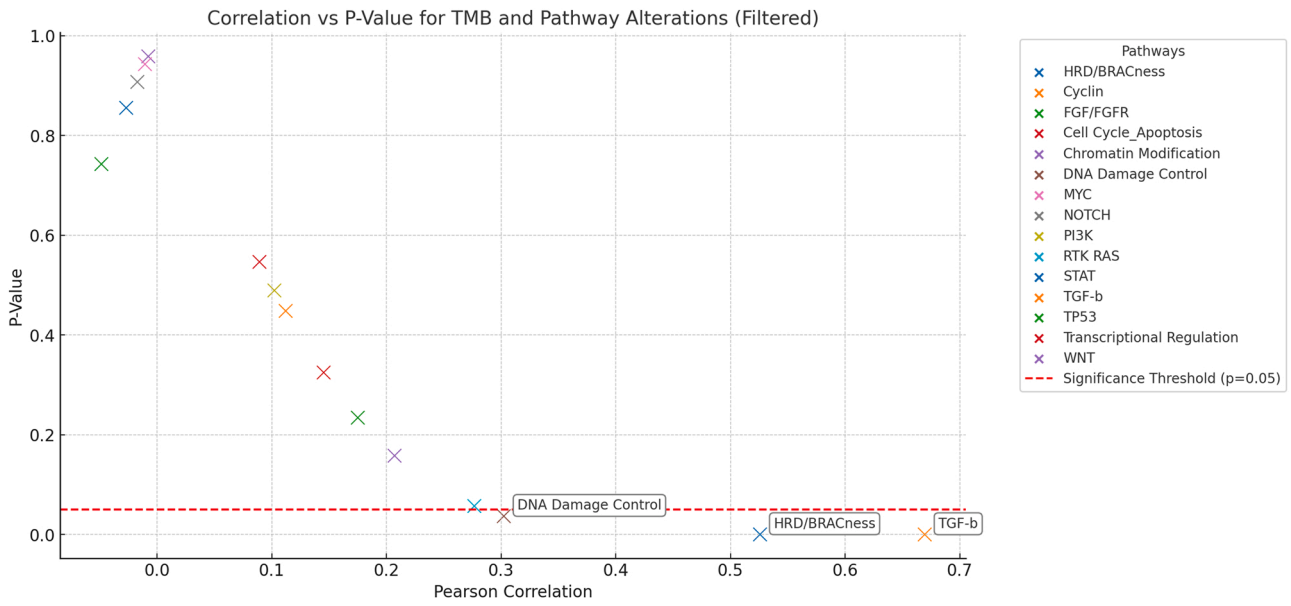

Fig. 7. Comparison of ORR and DCR in altered vs. non-altered pathways. The bar plot shows the percentage of patients achieving objective response rate (ORR) and disease control rate (DCR) in the altered and non-altered groups for each pathway.

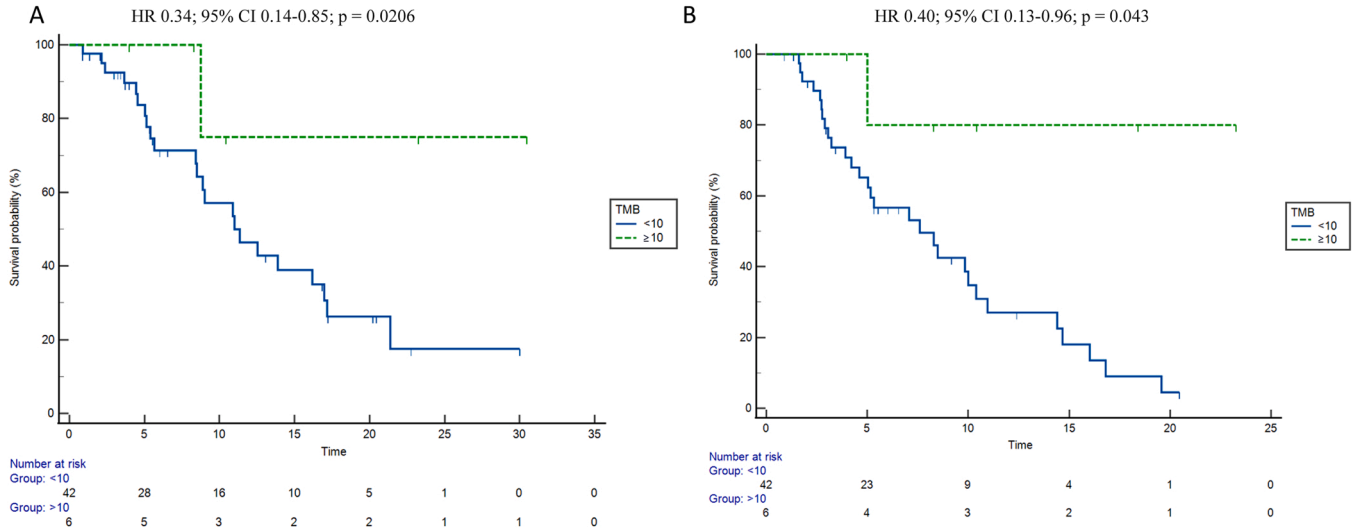

Fig. 8. Kaplan-Meier survival curves for overall survival (OS) and progression-free survival (PFS) in patients with high (>10 mut/Mb) versus low TMB. (A) OS in TMB-high vs. TMB-low patients. (B) PFS in TMB-high vs. TMB-low patients. Red lines indicate TMB-high groups, and blue lines indicate TMB-low groups.

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Ethics statement

The study was conducted in accordance with the Declaration of Helsinki and the protocol was approved by the Ethics Committee of each institution involved in the project. Under the condition of retrospective archival tissue collection and patients' data anonymization, our study was exempted from the acquisition of informed consent from patients by the institutional review board.

Institutional review board statement

The Ethical Review Board of each Institutional Hospital approved the present study. This study was performed in line with the principles of the Declaration of Helsinki.

Informed consent statement

Written informed consent for the treatment was obtained for all patients.

Disclosures

LR received consulting fees from AbbVie, AstraZeneca, Basilea, Bayer, BMS, Eisai, Elevar Therapeutics, Exelixis, Genenta, Hengrui,

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ACG reports consulting fees from AstraZeneca, Bayer, BMS, Eisai, Incyte, Ipsen, IQVIA, MSD, Roche, Servier; lecture fees from AstraZeneca, Bayer, BMS, Eisai, Incyte, Ipsen, Roche, Servier; travel expenses from AstraZeneca; research grants (to Institution) from AstraZeneca, Eisai.

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UM reports personal/consulting or speakers' bureau fees from AstraZeneca, Amgen, Boehringer Ingelheim, Diaceutics, Diatech, Eli Lilly & Company, GlaxoSmithKline, Hedra, Janssen Biotech, Merck, Merck Sharp & Dohme, Novartis, Roche Health Solutions Inc., and ThermoFisher Scientific outside the submitted work.

FP reports personal/consulting and/or speakers' bureau fees from Menarini International, Jansen, Thermofisher Scientifics and Roche outside the submitted work.

SL reports personal honoraria as invited speaker from Amgen, Astra Zeneca, Bristol-Myers Squibb, Incyte, GSK, Lilly, Merck Serono, MSD, Pierre-Fabre, Roche, Servier; participation in advisory board for Amgen, Astellas, Astra Zeneca, Bayer, Bristol-Myers Squibb, Daiichi-Sankyo, GSK, Incyte, Lilly, Merck Serono, MSD, Servier, Takeda, Rottapharm, Beigene, Fosun Pharma, Nimbus Therapeutics

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GWP Advisories and/or Speaker fees: Servier, Bayer, Roche Amgen, Merck, MSD, BMS, Sanofi, Lilly, Astra Zeneca, Astellas, Pierre-Fabre, Incyte, Arcus, CECOG

FF has received travel support from Ipsen, Abbvie, Astrazeneca and speaker's fees from AbbVie, MSD, Ipsen, Astrazeneca

LP Advisory role: AstraZeneca, Servier, Travel expenses: AstraZeneca, Ipsen.

GG Consulting Fees: Astra Zeneca, MSD, Servier, Seagen, Bayer, Amgen, Novartis, Ipsen, BMS

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JA received consulting fees from AstraZeneca, Jazz Pharmaceuticals, MSD, Roche, Servier, Taiho Oncology, Zymeworks; lecture fees from AstraZeneca, Roche, Servier; travel expenses from AstraZeneca, Roche, Servier.

AP reports personal fees for speakers and/or advisory role from Amgen, Merck Serono, MSD, BMS, Servier, Pierre-Fabre, BeOne, Daiichi Sankyo, Astra Zeneca, Takeda, Astellas, Nordic Pharma, Bayer.

GS Speaker/advisory: BMS, Janssen, Novartis, Roche, Bayer

GT received speaker fees (AstraZeneca)

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejca.2025.116035.

Data Availability Statement

The data underlying this article are not publicly available due to privacy and ethical restrictions. However, de-identified data can be requested from the corresponding author. Requests will be formally evaluated and approved by the Ethics Committee of San Raffaele Scientific Institute, in accordance with institutional and General Data Protection Regulation regulations. Requestors will be asked to provide contact details and a description of the intended use, for the purpose of logging and data use tracking. Data access will not be subject to discretionary approval by the study investigators.

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