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A WNT/β-catenin-associated transcriptional program predicts survival outcomes in patients with hepatocellular carcinoma

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is the most common form of liver cancer and a major cause of cancer-related deaths worldwide. Despite the wealth of genomics data, treatment and prognosis are still dependent on clinical and pathological factors. The molecular heterogeneity characterizing HCC is denoted by the different etiology and the related array of signaling pathways involved in tumor initiation and progression. Aberrant activation of the Wnt/β-catenin pathway is among the most frequent alterations in HCC, and stems from somatic mutations, functional over-activity of the transcription machinery and epigenetic cues.
Methods: Targeted DNA and RNA sequencing were combined to investigate the Wnt/β-catenin pathway and the effects of its dysregulation in the TCGA HCC cohort. For external validation, an independent cohort of78 Caucasian patients affected by HCC was used.
Results: We have identified a Wnt/β-catenin-related transcriptional signature denoting pathway activity regardless of the presence of pathway-related activating mutations. This model predicts survival outcomes in two independent cohorts of HCC patients (TCGA cohort, $N = 177$; Rome cohort, N78) and is associated with distinctive immunogenomic features.
Conclusions: A non-genetic state recapitulating the transcriptional footprint associated with *CTNNB1* mutation identifies wild-type HCCs characterized by unfavorable survival outcomes and immune-excluded tumor immune microenvironment.

Abbreviations: HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; APC, Adenomatous polyposis coli; GSK3β, Glycogen synthase kinase 3β; CK1α, casein kinase 1 isoform-α; PP2A, Protein phosphatase 2; LRP5, low-density-lipoprotein receptor-related protein 5; LRP6, low-density-lipoprotein receptor-related protein 6; Dvl, Dishevelled; TCF/LEF T, cell factor/lymphoid enhancer-binding factor; HIF-1α, hypoxia-inducible factor 1α; FOXO, forkhead box protein O; SOX, sex-determining region Y box; OS, overall survival; DFS, disease-free survival; GO, Gene ontologies.

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1. Introduction

Hepatocellular carcinoma (HCC) is the predominant form of liver cancer and one of the most common malignancies worldwide (Vogel et al., 2022). The therapeutic landscape of HCC (e.g.; surgery; locoregional treatments; targeted agents and immunotherapy) is mostly dependent on clinical features that provide information on preserved liver function. Indeed; while large-scale sequencing programs have shed light on the most common genetic alterations in HCC; molecular biomarkers have not yet been implemented in clinical practice (Gordan et al., 2020; Llovet et al., 2021).

The Wnt/β-catenin signaling pathway is a key regulator of liver homeostasis and regeneration, while its aberrant activation, which is prevalently related to β-catenin (*CTNNB1*) mutations, is a common alterations in HCC (Perugorria et al., 2019). The Wnt signaling is regulated by the cascade effector β-catenin. In the absence of ligand stimulation; β-catenin is engaged in a multiprotein destruction complex that includes Axin1; Adenomatous polyposis coli (APC); Glycogen synthase kinase 3β (GSK3β); casein kinase 1 isoform-α (CK1α); Protein phosphatase 2 A (PP2A) and the E3-ubiquitin ligase β-TrCP. This multiprotein complex mediates β-catenin degradation via the proteasome machinery. The pathway is activated following the binding of secreted Wnt ligands to Frizzled receptors and low-density-lipoprotein receptor-related proteins 5 and 6 (LRP5 and LRP6) co-receptors; which triggers the recruitment of Dishevelled (Dvl) proteins to the plasma membrane. Dvl polymers inactivate the destruction complex; thus leading to β-catenin stabilization and translocation to the nucleus; where it recruits co-activators and transcription factors (Nusse and Clevers, 2017; Zhan et al., 2017). Although the nuclear DNA-binding T cell factor/lymphoid enhancer-binding factor (TCF/LEF) represents its main partner (Cadigan and Waterman, 2012); β-catenin can also bind to other partners; including hypoxia-inducible factor 1α (HIF-1α); forkhead box protein O (FOXO); and sex-determining region Y box (SOX) transcription factors (Kaidi et al., 2007; Essers et al., 2005; Kormish et al., 2010). The resulting complexes promote the transcription of a high number of target genes involved in the regulation of several biological processes, including cell cycle progression and proliferation, inhibition of apoptosis, cell growth and migration (Vlad et al., 2008).

Activation of the Wnt/β-catenin pathway is an early event in HCC pathogenesis (Suzuki et al., 2002; Fujie et al., 2001) and it is implicated in HCC progression, stemness, metastasis and drug resistance (Khalaf et al., 2018; Yamashita et al., 2007; Liu et al., 2010; Liao et al., 2020; Liu et al., 2015). Altogether, these data support the multi-step involvement of Wnt/β-catenin in liver carcinogenesis. More recently, upregulated Wnt/β-catenin signaling was linked to immune evasion and resistance to immune checkpoint inhibitors (ICIs) in HCC mouse models (Ruiz de Galarreta et al., 2019).

Massive characterization efforts have shed light on the genetic underpinnings of constitutive β-catenin activation. For instance, the Cancer Genome Atlas Research (TCGA) Network described gain-of-function (GOF) C*TNNB1* mutations in 27% of HCC patients, whereas mutations in the components of the β-catenin destruction complex *APC* and *AXIN1* are found in 3% and 8% of HCC, respectively (Cancer genome atlas research network, 2017). While the frequencies of genetic deregulations cannot fully account for the striking prevalence of β-catenin signaling activation in HCC (Bengochea et al., 2008); non-genetic mechanisms have been described that contribute to aberrant activation. These include chromatin-remodeling mechanisms (Barker et al., 2001; Carotenuto et al., 2017) and deregulated expression of microRNAs and long noncoding RNAs (Rana et al., 2019; Klingenberg et al., 2017; Braconi et al., 2011). In addition, ethnicity-specific differences exist in HCC patients in terms of pathogenesis and survival outcomes (Llovet et al., 2021).

Reasoning that an integrative pathway-focused analysis may provide a more detailed picture on how an established oncogenic route impacts clinical outcomes, we characterized the Wnt/β-catenin pathway at the transcriptional and genetic level. To this end, we matched targeted DNA and RNA sequencing to characterize the impact of pathway deregulation in Caucasian patients from the TCGA HCC cohort. A cohort of 78 patients who underwent surgical resection at the IRCCS Regina Elena National Cancer Institute (Rome cohort) was used for external validation. The workflow of the study is reported in Fig. 1.

2. Methods

2.1. Cohorts and patients

For the development of the WNT signature, transcriptomic and mutational data from two independent cohorts of HCC patients were analyzed. Data related to the TCGA HCC study ($N = 177$) were downloaded from cBioPortal (Cerami et al., 2012; Gao et al., 2013) and https://www.cbioportal.org). The Rome cohort included 78 HCC patients who underwent surgical resection at the IRCCS Regina Elena National Cancer Institute. Only Caucasian patients were selected from the TCGA dataset to align with the demographic characteristics of our internal cohort and minimize potential confounding due to ethnic variability in genetic and environmental factors. This approach ensured homogeneity within the cohort and allowed for a more consistent analysis of the association between the WNT score and survival outcomes/immunogenomic features. Written informed consent was obtained from all participants in the Rome cohort. This study was conducted in accordance with the Declaration of Helsinki and approved by the IRE-IFO Institutional Ethics Committee (Approval number: CEC/532/15). We did not use any variant interpreter for predicting mutation pathogenicity.

2.2. Next generation sequencing

Genomic DNA and RNA were extracted from 5 μm FFPE tissue sections using the AllPrep DNA/RNA FFPE kit (Qiagen, Valencia, CA, USA). The concentration and integrity of purified DNA/RNA samples were assessed by Qubit fluorometer (Thermo Fisher Scientific, Hampton, NH, USA) and 2200 TapeStatio (Agilent Technologies, Santa Clara, CA, USA), respectively. The TruSeq Custom Amplicon Kit was used for the DNA library preparation. The custom panel included 17 selected genes (*AMER1*, *APC*, *CTNNB1*, *FBXW7*, *SOX9*, *TCF7L2*, *ACVR1B*, *SMAD2*, *SMAD4*, *PIK3CA*, *PTEN*, *BRAF*, *MAP3K21*, *KRAS*, *NRAS* and *TP53*) and was designed using Illumina® DesignStudio™. Samples were sequenced on an Illumina NextSeq 500 (Illumina, Inc., San Diego, CA, USA) in a paired-end mode, sequencing 150 bp from each side (De Nicola et al., 2018). The RNA-Seq library was prepared using the AmpliSeq for Illumina RNA Wnt panel (including 169 genes involved in the regulation of the Wnt/β-catenin signaling pathway) according to the manufacturer's instructions. Pooled sequencing libraries were sequenced in 150-bp paired-end reads on Illumina MiSeq sequencer (Illumina).

2.3. Statistical and bioinformatic analyses

Statistical and bioinformatic analyses were performed using R (version 4.5.2). Survival curves were estimated using the Kaplan–Meier method and compared using the log-rank test. Multivariable Cox proportional hazards models were constructed including clinicopathological variables available in the datasets and potentially impacting survival outcomes. Hazard ratios (HR) and corresponding 95% confidence intervals (CI) were reported. Comparisons between two groups were performed using the Wilcoxon rank-sum test. Associations between continuous variables were assessed using Pearson correlation analysis. Unless otherwise specified, statistical significance was defined as $P < 0.05$. Differential gene expression analyses were conducted using the DESeq2 R package, which models count data using a negative binomial distribution (Love et al., 2014). Genes differentially expressed between *CTNNB1*-mutant and *CTNNB1*-wt tumors were identified using Wald

tests; and multiple testing correction was performed using the Benjamini–Hochberg method to control the FDR. Functional interpretation of gene expression profiles was performed using several complementary approaches. Gene ontology (GO) biological process enrichment was summarized and visualized using the rrvgo R package (https://ssayols.github.io/rrvgo). Pathway activity was estimated using Pathway Responsive Genes (PROGENy); which infers the activity of 14 canonical signaling pathways based on downstream transcriptional targets derived from perturbation experiments (Schubert et al., 2018). Transcription factor activity was inferred using the DoRothEA framework; which estimates regulatory activity based on curated transcription factor–target interaction networks (Garcia-Alonso et al., 2019). Immunogenomic data related to the TCGA HCC cohort were obtained from the CRI iAtlas Portal (https://www.cri-iatlas.org/resources/). In addition; tumor samples were classified according to the pan-cancer tumor microenvironment classification system; which stratifies tumors into four immunological subtypes: immune-enriched fibrotic; immune-enriched non-fibrotic; immune-depleted fibrotic; and immune-depleted non-fibrotic

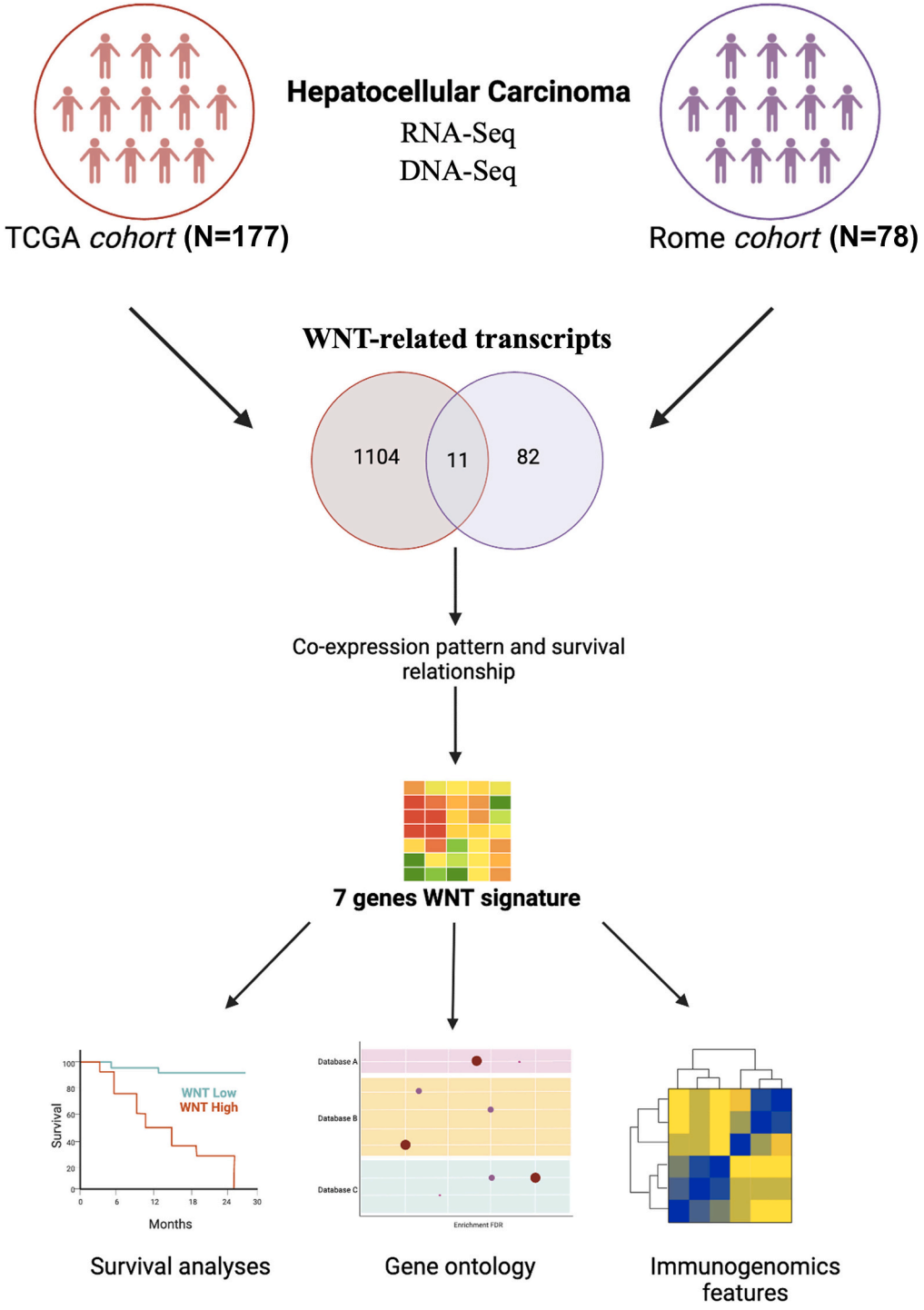

Fig. 1. Study design. RNA-Seq data from the TCGA and Rome cohorts were used to identify shared upregulated genes in the *CTNNB1*-mutant background. A pool of 7 WNT-related genes was extracted on the basis of co-expression and relationship with survival outcomes. The resulting WNT signature was tested in survival analyses. Immunological correlates were also investigated.

(available at http://science.bostongene.com/tumor-portrait) (Cerami et al., 2012).

2.4. WNT transcriptional signature

To derive the WNT transcriptional signature, we applied a stepwise strategy integrating cross-cohort reproducibility and biological coherence. Genes significantly upregulated in *CTNNB1*-mutant tumors in the TCGA cohort were intersected with genes upregulated in the Rome cohort, resulting in a shared set of 11 transcripts consistently associated with *CTNNB1* mutation status. From this pool, five genes (*NKD1*, *TCF7*, *AXIN2*, *BMP4* and *CTNNB1*) were selected as core components based on strong co-expression patterns and their established roles as canonical components or downstream effectors of Wnt/β-catenin signaling. Two additional genes (*DVL1* and *RUVBL1*) were included because they were associated with overall survival in the TCGA cohort and have documented mechanistic roles in Wnt pathway regulation. For each sample, the WNT signature score was calculated as the unweighted mean expression of the seven selected genes. Gene expression values were previously normalized and centered, allowing negative values; therefore, averaging gene expression provided a direct measure of coordinated pathway activity without introducing weighting parameters that could increase the risk of overfitting. Tumors with a mean WNT score ≥ 0 were classified as WNT high, indicating above-average pathway activity relative to the cohort distribution, whereas tumors with scores <0 were classified as WNT low.

3. Results

3.1. Identification of a prognostic Wnt/β-catenin-associated transcriptional signature

To assess the activation status of the Wnt/β-catenin signaling pathway, recognizing that such activation can occur through multiple mechanisms beyond *CTNNB1* mutations, we adopted a transcriptional-level approach based on the analysis of two independent datasets. First, we performed differential gene expression analysis to extract the transcriptional output associated with *CTNNB1* mutations in the TCGA HCC cohort ($N = 177$). Given the impact of ethnicity on the pathogenesis of HCC, only Caucasian patients were included (Fig. 2A). Next, we performed pathway-targeted RNA-Seq on the Rome cohort, which included 78 HCC patients surgically treated at the IRCCS Regina Elena National Cancer Institute (Rome cohort). For this last analysis, we leveraged a Wnt panel encompassing 169 Wnt-associated genes. The

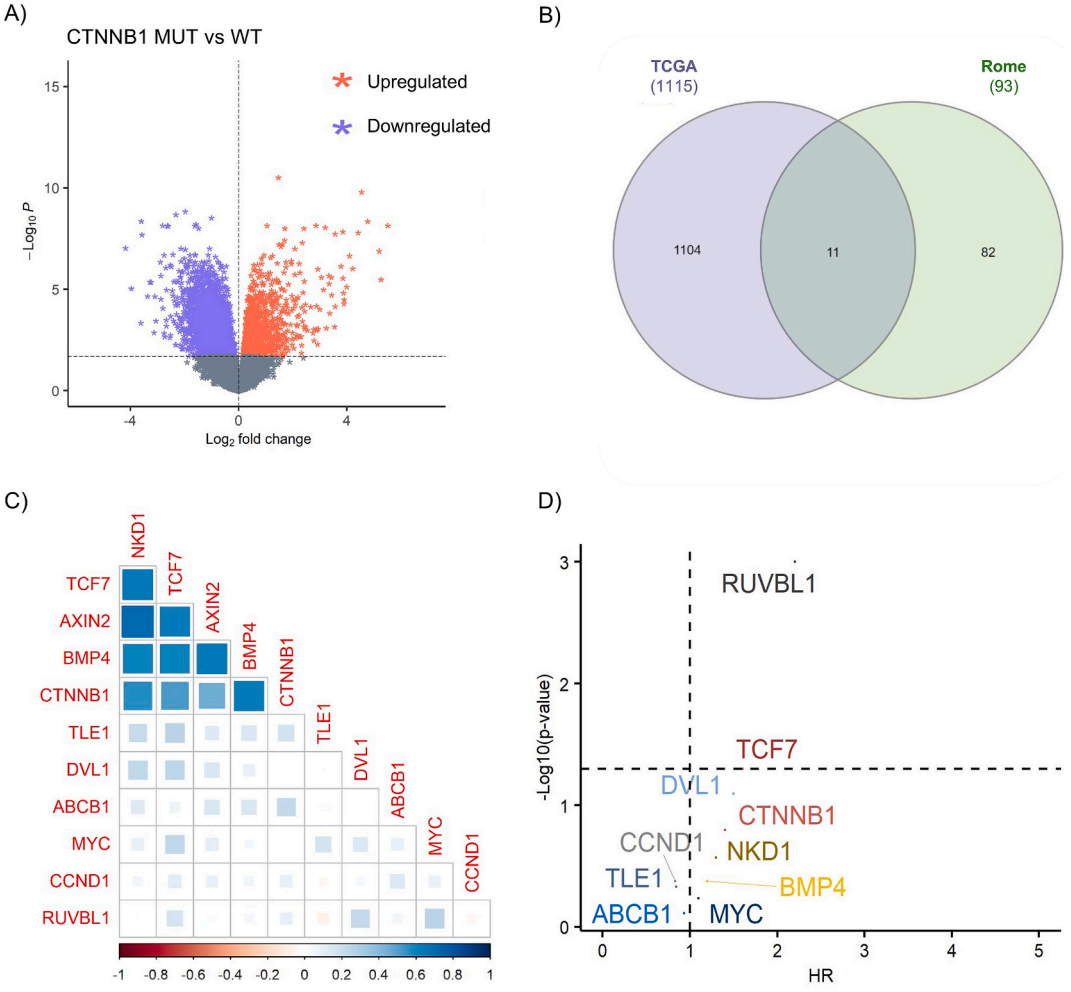

Fig. 2. Identification of the WNT transcriptional signature. A. Volcano plot of differentially expressed genes in relation to *CTNNB1* status in the TCGA HCC cohort. **B.** Venn diagram of enriched genes: the purple circle includes the number of upregulated transcripts in *CTNNB1*-mutated versus *CTNNB1*-wt HCCs from the TCGA cohort, whereas the number in the green circle indicates the Wnt-related, upregulated transcripts evaluated in the Rome cohort. The intersection (11 genes) represents upregulated genes in the TCGA that were also evaluated in the targeted RNA-seq (Rome cohort) **C.** Heatmap showing the co-expression pattern of selected transcripts (Pearson correlation coefficient). **D.** Scatter plot showing univariate Cox regression analyses for OS of selected transcripts in the TCGA HCC study. Abbreviations: HCC, hepatocellular carcinoma; HR, hazard ratio; MUT, mutated; OS, overall survival; TCGA, the cancer genome atlas; WT, wild type. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

baseline characteristics of patients included in the Rome cohort are reported in Table 1. Up-regulated transcripts in the *CTNNB1*-mutant background from the TCGA HCC cohort (1104 transcripts) were intersected with upregulated transcripts assessed in the Rome cohort (82 genes) to generate a cross-cohort core of 11 genes (Fig. 2B). Within this restricted set, we prioritized transcripts that not only displayed coherent co-expression patterns within the Wnt/β-catenin pathway but also reflected established biological roles in pathway-driven transcriptional programs. Five genes (*NKD1*, *TCF7*, *AXIN2, BMP4* and *CTNNB1*) fulfilled these criteria, representing canonical components or direct effectors of the pathway and showing tightly correlated expression profiles (Fig. 2C). To further enrich the signature with clinical relevance, we incorporated two additional genes (*DVL1* and *RUVBL1*) that showed an association with overall survival in the TCGA cohort (*DVL1*, HR 1.5, 95% CI 0.95–2.4, $p = 0.08$ and *RUVBL1*, HR 2.2, 95% CI 1.4–3.5, $p = 0.001$, Fig. 2D) and have documented mechanistic links to Wnt/β-catenin signaling. This integrative selection process yielded a robust 7-gene signature, which we designated as the WNT signature, capturing the transcriptional state associated with Wnt/β-catenin pathway activation.

Several components of the WNT signature displayed higher expression in HCC tissues compared with normal liver samples in the TCGA cohort. Although individual genes showed variable degrees of upregulation, the collective behavior of the seven-gene panel revealed a coherent increase in pathway activity across tumors. This coordinated trend supports the notion that the signature captures a biologically meaningful transcriptional state associated with Wnt/β-catenin activation in liver carcinogenesis (Supplementary Fig. 1).

We next investigated the prognostic significance of the 7-gene WNT signature in the TCGA Caucasian HCC cohort. Patients with tumors expressing higher levels of the signature (WNT high) had significantly shorter overall survival (OS) and disease-free survival (DFS) compared with those characterized by low expression (WNT low) (OS log-rank $P = 0.0046$; Fig. 3A; DFS log-rank $P = 0.0093$; Fig. 3B). Beyond the expected enrichment of *CTNNB1* mutations, WNT high tumors also showed a significantly higher frequency of *TP53* mutations compared with WNT low tumors. Analysis of the most recurrently mutated HCC genes in the TCGA cohort further revealed an enrichment of *ROBO1* mutations in the WNT high subgroup. As *ROBO1* is not a canonical component of the Wnt/β-catenin pathway, this pattern likely reflects broader co-mutational features of Wnt/β-catenin-activated tumors rather than a pathway-specific interaction (Fig. 3C). No additional patterns of co-occurrence or mutual exclusivity were observed among the most frequently mutated HCC genes, indicating the absence of somatic interactions that could account for the phenotype (Fig. 3D).

Table 1
Characteristics of HCC patients included in the Rome cohort ($N = 78$).

Characteristics	N (%)
Age at diagnosis	
Median (IQR)	68 (40–88)
Gender	
Male	53 (67.9%)
Female	25 (32.1%)
BCLC stage	
0-A	41 (52.6%)
B	37 (47.4%)
Cirrhosis	
No	16 (20.5%)
Yes	62 (79.5%)
Child-Pugh class	
A	74 (94.9%)
B	4 (5.1%)
Viral hepatitis infection	
HCV, HBV, or both	49 (62.8%)
None	29 (37.2%)

BCLC, Barcelona Clinic Liver Cancer; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

In the Rome cohort, patients with WNT high tumors had shorter OS compared with those with WNT low disease (log-rank $P = 0.014$, Fig. 4A). In a multivariable Cox regression model for OS, which included key clinical and molecular variables (such as tumor stage and viral infection), the WNT signature remained significantly associated with OS, confirming its role as an independent prognostic factor (Fig. 4B). Moreover, we did not find any significant association between the WNT signature and baseline clinical characteristics of the patients in the Rome cohort (Fig. 4C), further supporting its role as a distinct and independent prognostic marker.

To further assess the robustness of the gene-selection strategy, we performed additional sensitivity analyses comparing the prognostic performance of the 5-gene core set, the 7-gene WNT signature, and the full 11-gene set initially identified from the cross-cohort intersection. In both the TCGA and Rome cohorts, the 5-gene core set retained a consistent survival trend, whereas inclusion of all 11 genes attenuated the prognostic signal (Supplementary Fig. 2). These results confirm that the 7-gene signature provides the strongest and most stable prognostic discrimination across datasets.

We also evaluated the WNT score as a continuous variable in Cox proportional hazards models. Increasing WNT score was associated with progressively higher hazard ratios for OS in both the TCGA and Rome cohorts, confirming that the prognostic effect of the signature is maintained independently of any specific cutoff (Supplementary Fig. 3).

Taken together, these findings indicate that the WNT signature captures a reproducible transcriptional program that reflects Wnt/β-catenin pathway activation beyond the presence of *CTNNB1* mutations, delineating a biologically and clinically meaningful subset of HCCs characterized by heightened Wnt/β-catenin activity.

3.2. *The WNT signature predicts inferior survival outcomes regardless of the presence of CTNNB1 mutations*

The extent to which mutation-independent events contribute to aberrant Wnt/β-catenin signaling remains elusive. To explore the relationship between *CTNNB1* status and the WNT signature in terms of outcome prediction, we challenged our model by further stratifying WNT high patients based on the presence or absence of *CTNNB1* mutations and including this stratification in survival analyses. In the TCGA Caucasian HCC cohort, the association of the WNT signature with worse OS was similar in the WNT high/*CTNNB1*-mutated and WNT high/*CTNNB1*-wt subgroups (WNT high/*CTNNB1*-mutated versus WNT low log-rank $P = 0.0092$; WNT high/*CTNNB1*-wt versus WNT low log-rank $P = 0.0495$; Fig. 5A). A comparable trend was observed in the Rome cohort, albeit the limited size of the subgroups compared partially hindered this analysis (Fig. 5B).

The distribution of *CTNNB1* mutations within the WNT high and low subsets in both cohorts is reported in Fig. 5C, and indicated that a non-negligible fraction of *CTNNB1* mutations are transcriptionally silent. Moreover, differential gene expression analysis comparing WNT high/*CTNNB1*-mutated and WNT high/*CTNNB1*-wt tumors retrieved only a limited number of differentially expressed genes, consistent with a largely overlapping molecular landscape (Fig. 5D). These findings indicate that tumors classified as WNT high share a common Wnt/β-catenin-activated transcriptional program irrespective of *CTNNB1* mutation status, reflecting the ability of the WNT signature to identify a subset of *CTNNB1*-wt tumors that adopt a molecular profile closely resembling *CTNNB1*-mutant cases.

Thus, our data indicate that deregulated Wnt/β-catenin transcriptional program is associated with inferior survival outcomes regardless of the presence of *CTNNB1* mutations, and therefore that gene expression outperforms somatic mutations in predicting clinical outcomes.

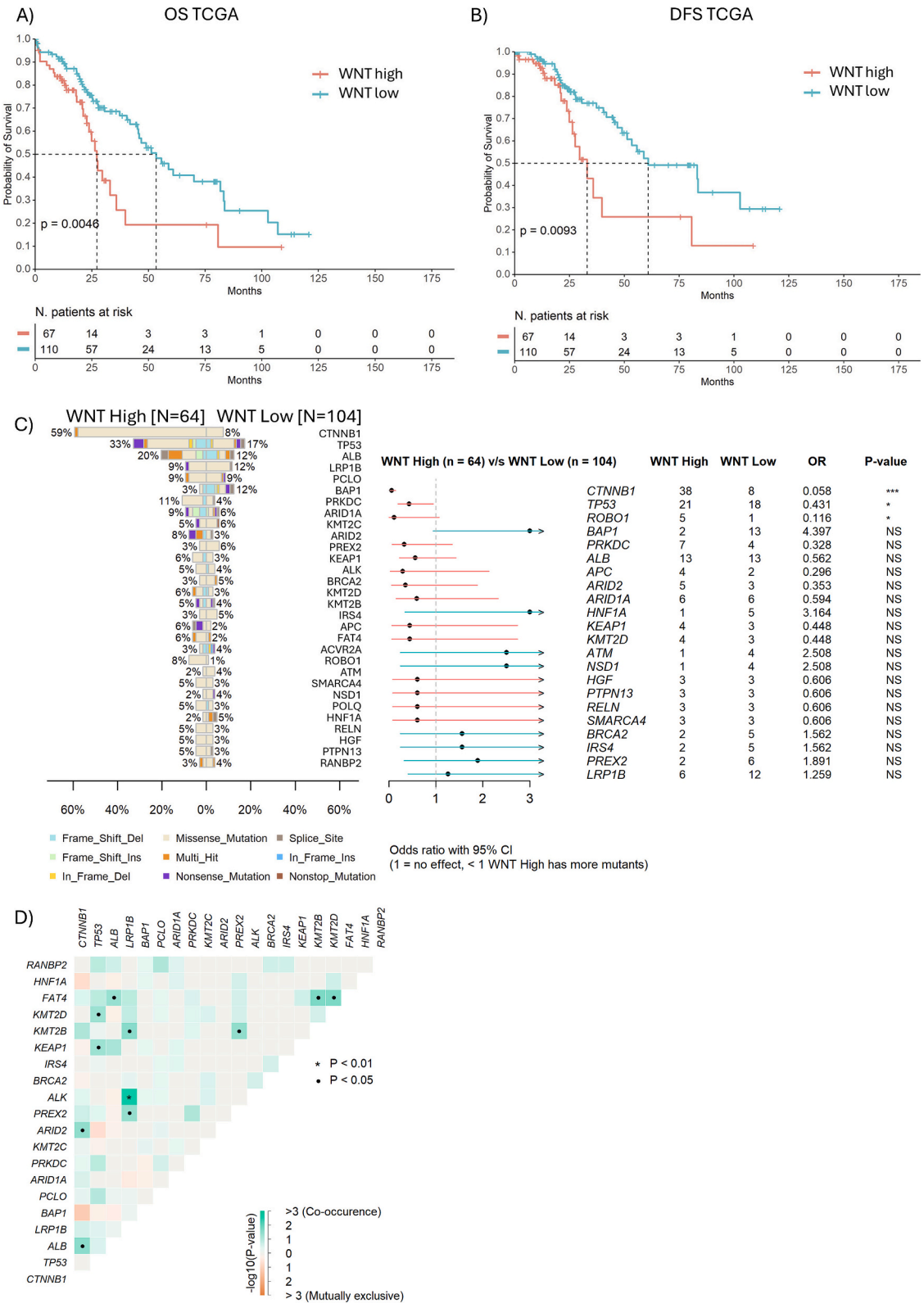

Fig. 3. Survival analyses and mutational frequencies in the TCGA HCC cohort. A, B. Kaplan-Meier survival curves for OS (**A**) and DFS (**B**) comparing patients with WNT high versus WNT low HCC. **C.** Mutation frequencies of the most recurrently mutated HCC genes in the TCGA cohort, shown separately for the WNT high and WNT low subgroups (left panel); forest plot of odds ratio (OR) comparing mutation frequencies of top mutated genes between the WNT high and WNT low groups. An OR < 1 indicates a higher mutation frequency in the WNT high group. Significant comparisons ($p < 0.05$) are indicated with asterisks (right panel). **D.** Heatmap showing co-occurrence/mutual exclusivity of recurrently mutated genes in HCC. Asterisks denote $p < 0.01$, while circles denote $p < 0.05$.
Abbreviations: DFS, disease-free survival; HCC, hepatocellular carcinoma; OS, overall survival; TCGA, the cancer genome atlas.

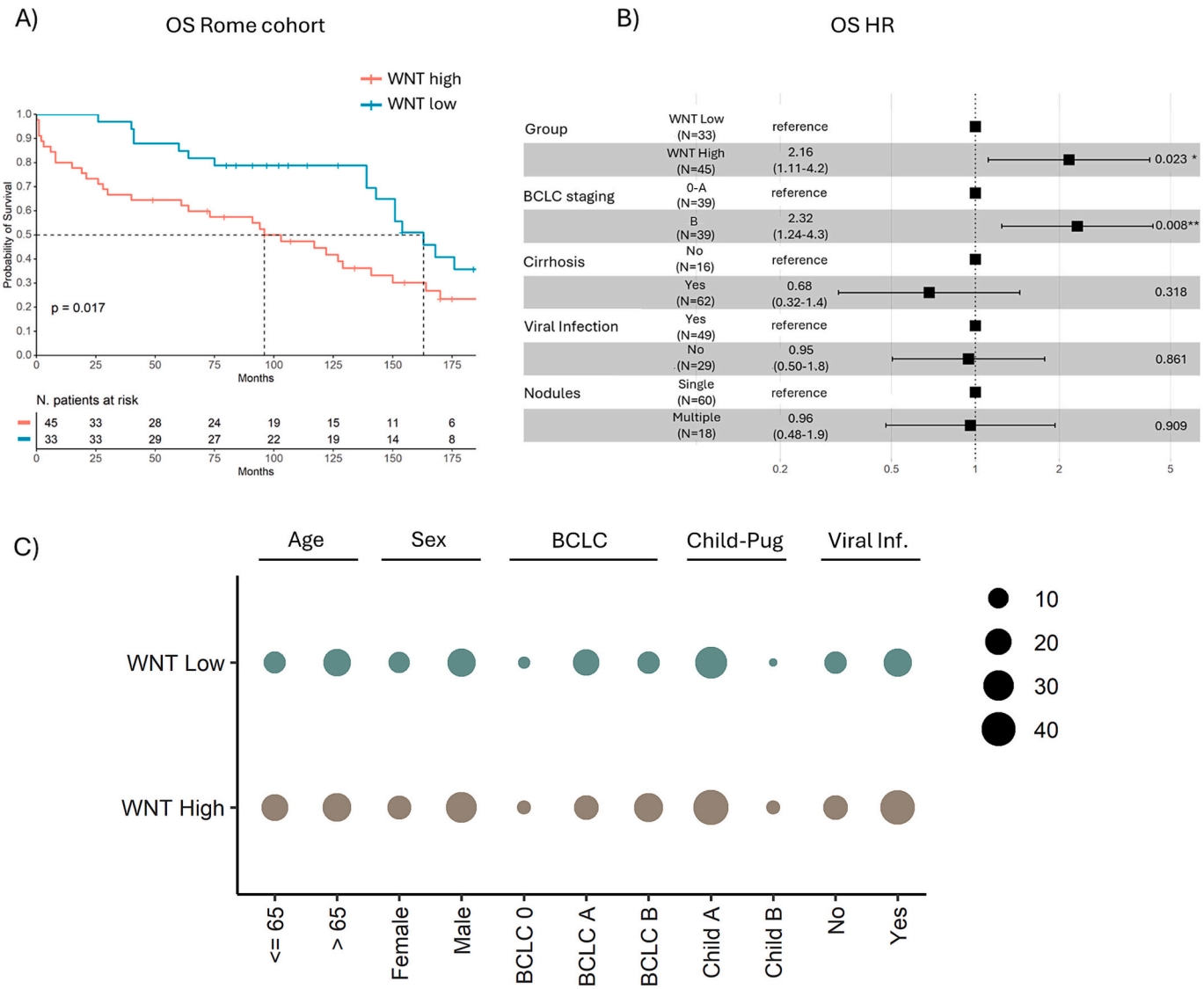

Fig. 4. Survival analyses in the Rome cohort. A. Kaplan-Meier survival curve for OS comparing patients with WNT high versus WNT low HCC. **B.** Multivariable Cox regression analysis. **C.** Bubble chart showing the distribution of clinical features in the WNT high and WNT low subsets. No statistically significant differences were reported.
Abbreviations: BCLC, Barcelona-clinic liver cancer; HR, hazard ratio; OS, overall survival.

3.3. Molecular correlates of deregulated Wnt/β-catenin transcriptional program

To investigate differences in cancer-intrinsic and immune-related features between WNT high and low subgroups, we evaluated transcriptomic data from the TCGA Caucasian HCC cohort. Differential gene expression analysis (WNT high vs WNT low) is shown in Fig. 6A. Gene ontologies (GO) confirmed the enrichment of biological processes related to Wnt/β-catenin pathway in the WNT high subset (Fig. 6B). In transcription factor analysis (DoRothEA), WNT high tumors showed lower enrichment scores of immune-related transcription factors (STAT1/ZHX2) compared to WNT low cases (Fig. 6C). Likewise, the WNT high group was associated with higher enrichment scores of the downregulator of chemokine receptors ZNF175 and the expected TCF7. Consistently, the analysis of pathway-level signatures (PROGENy) revealed lower enrichment scores of immune-associated pathways in WNT high as compared to WNT low HCCs (transforming growth factor-β, nuclear factor-κB, tumor necrosis factor-α) (Fig. 6D). Conversely, the pro-proliferative MAPK pathway and pro-angiogenic VEGF signature were enriched in the WNT high group (Fig. 6D).

Recently, four distinct pan-cancer immunogenomic subtypes have been described: 1) immune depleted and non-fibrotic (D), 2) immune depleted and fibrotic (F), 3) immune-enriched, non-fibrotic (IE), and 4) immune-enriched, fibrotic (IE/F) (Bagaev et al., 2021). We observed that a higher proportion of WNT high tumors were characterized by an immune-depleted and non-fibrotic phenotype (D subtype) compared with the WNT low group (Fig. 7A, left graph). To more exactly explore the proportion of each immunogenic subtype in WNT high and WNT low tumors, we found that WNT low tumors had a significantly higher proportion of immune-enriched subtypes compared to WNT high tumors, while WNT high tumors tended to be more frequently associated with the immune-depleted, non-fibrotic (D) subtype (Fig. 7A, right graph). To assess whether these microenvironmental differences persist within *CTNNB1*-mutated tumors, we performed a stratified analysis restricted to this subgroup. Within *CTNNB1*-mutated cases, WNT high tumors showed a descriptive trend toward enrichment of immune-depleted phenotypes compared with WNT low tumors, although this difference did not reach statistical significance, likely due to the limited sample size (Supplementary Fig. 4).

Accordingly, the analysis of immune-related signatures used to

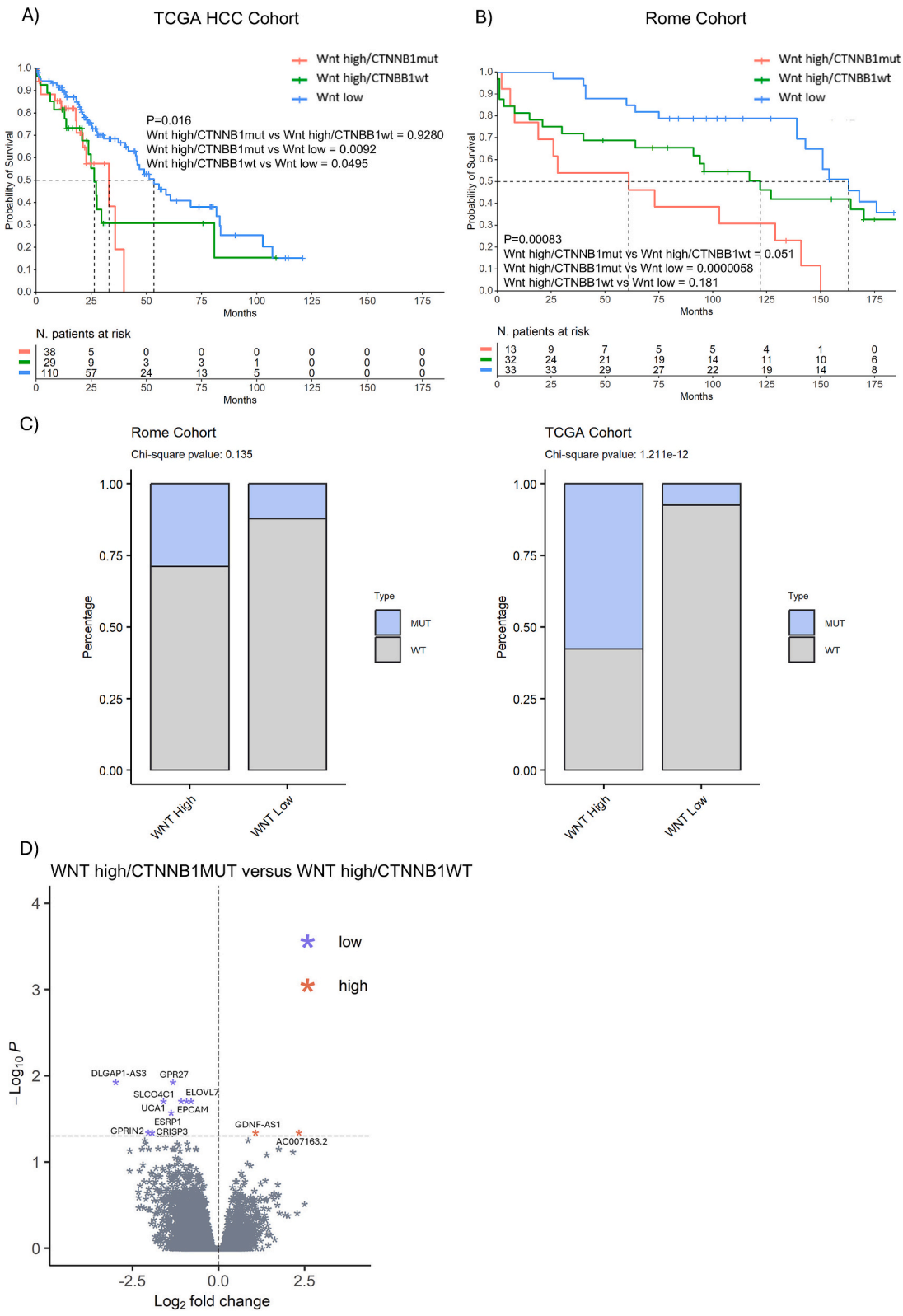

Fig. 5. Survival analyses according to *CTNNB1* mutational status and frequencies of the WNT signature in the TCGA and Rome cohorts. A, B. Kaplan-Meier survival curves for OS comparing WNT high HCC divided according to *CTNNB1* status and WNT low HCC in the TCGA (**A**) and Rome (**B**) cohorts. **C.** Bar charts summarizing the distribution of *CTNNB1* mutations among the investigated subgroups in the TCGA and Rome cohorts. Statistical significance was assessed using the chi-square test. **D.** Volcano plot showing differentially expressed genes in the WNT high/*CTNNB1*-mutated subgroup versus the WNT high/*CTNNB1*-wt subset in the TCGA cohort.

Abbreviations: HCC, hepatocellular carcinoma; MUT, mutated; OS, overall survival; TCGA, the cancer genome atlas; WT, wild type.

Fig. 6. Transcription factors and pathway-level analyses in the TCGA HCC study. A. Volcano plot of differential gene expression analysis when comparing WNT high versus low HCC (TCGA). **B.** Gene Ontology analysis illustrating biological processes enriched in the WNT high subgroup. **C, D.** Waterfall plots illustrating transcription factor activity (DoRothEA) (**C**) and PROGENy pathway signatures (**D**) in the WNT high versus WNT low subgroup.

Abbreviations: EDA, ectosysplasin A; FDR, false discovery rate; FZD, frizzled; GPCR; G protein-coupled receptor; HCC, hepatocellular carcinoma; KEGG, Kyoto encyclopedia of genes and genomes; LRP5, low-density-lipoprotein receptor-related protein 5; NES, normalized enrichment score; TCF, T-cell factor; TCGA, the cancer genome atlas; SLC, solute carrier.

generate two independent immune subtypes (Bagaev et al., 2021; Thorsson et al., 2018) revealed differences among the the WNT high and WNT low subgroups. Notably, WNT high tumors exhibited a reduction in lymphocyte infiltration and antitumor cytokine signatures, supporting their classification as predominantly immune-excluded tumors (**Fig. 7B** and **C**). Accordingly, the analysis of key immunomodulators in the TCGA Caucasian HCC cohort revealed a lower *CD274* mRNA expression (the gene encoding for PD-L1) in the WNT high subset compared with the WNT low group, and a similar pattern was observed for other immunomodulators (Fig. 7D and **Supplementary Fig. 5**). Overall, these finding indicate that the WNT signature defines a subgroup of tumors with reduced immune infiltration, a finding that is of particular relevance given the recent success of immune checkpoint inhibitors in HCC (Rizzo et al., 2021; Finn et al., 2020).

4. Discussion

Aberrant activation of Wnt/β-catenin signaling is common in HCC. While the contribution of somatic mutations to pathway hyperactivation is well established, several non-genetic events have been identified that explain the gap between frequencies of activating mutations and prevalence of pathway dysregulation. Among these, Wnt/β-catenin-activating epigenetic mechanisms seem to play a key role given the involvement of chromatin remodeling in the pathogenesis of HCC and the high rate of mutations in chromatin regulators reported in other studies (Fujimoto et al., 2012; Braghini et al., 2022). Nevertheless, the clinical implications of deregulated Wnt pathway and increased β-catenin activity remain elusive.

Here, we profiled a cohort of78 surgically treated HCC patients by combining targeted RNA and DNA sequencing to explore the relationship between Wnt/β-catenin-driven transcriptional program and survival outcomes. In this way, we identified a 7-genes transcriptional signature (WNT signature) denoting Wnt/β-catenin aberrant activation and predicting unfavorable survival outcomes in HCC patients. Also, we found that the co-expression pattern of Wnt-related genes is associated with distinctive immunogenomic features, overall indicating an association between aberrant Wnt/β-catenin signaling and immune exclusion. This finding is in agreement with previous evidence from HCC mouse models showing that *CTNNB1*-mutated HCCs fail to produce CCL5 chemokine which is responsible for attracting dendritic cells within the mouse tumor (Ruiz de Galarreta et al., 2019). Accordingly; preliminary clinical observations suggested that Wnt/β-catenin signaling activation promotes immune escape; and thus resistance to anti-PD-1 therapies in patients (Harding et al., 2019). Within *CTNNB1*-mutated tumors; the association between higher WNT activity and a more immune-depleted microenvironment appeared directionally consistent with our main findings; although confirmation in larger cohorts with sufficient statistical power will be required. Notably; *CTNNB1*-mutated tumors classified as WNT low likely reflect biologically non-functional or weakly activating *CTNNB1* alterations; a phenomenon already described in HCC where not all *CTNNB1* mutations translate into effective pathway activation. This observation reinforces the value of a transcription-based readout; as it discriminates between functionally active and inactive *CTNNB1* events and more accurately captures the downstream biological state relevant to immune exclusion. These observations reinforce the notion that Wnt/β-catenin-driven biology in HCC extends well beyond the presence of *CTNNB1* mutations. The transcriptional program captured by the WNT signature reflects a broader Wnt/β-catenin-associated state that can arise through multiple genetic and non-genetic mechanisms; thereby identifying a biologically coherent subgroup of tumors that phenocopy *CTNNB1*-mutant HCCs despite being genetically wild-type. This concept aligns with accumulating evidence that pathway activation in HCC is frequently sustained by epigenetic remodeling; altered transcriptional regulation; and microenvironmental cues rather than by mutations alone (Liu et al., 2010; Kaur et al., 2012; Sharma et al., 2021). Importantly, the WNT signature also identified a subset of patients with increased Wnt/β-catenin signaling despite the absence of *CTNNB1* mutations.

While we acknowledge that our findings have some limitations (e.g., retrospective design), we believe that our results may have important implications for more personalized treatments. ICIs have recently proven efficacy in the treatment of patients with HCC (Rizzo et al., 2021; Finn et al., 2020). However, the identification and clinical validation of predictive biomarkers of ICIs efficacy remains a challenge in several tumor types, including HCC. Indeed, only a limited proportion of HCC patients benefit from these therapies. Provided that the involvement of Wnt/β-catenin activation in resistance to ICIs needs confirmation in prospective and adequately powered studies, our WNT signature holds the potential to serve as a predictive biomarker of low response to ICIs. Indeed, while approximately 35% of HCC tumors carry somatic mutations leading to Wnt/β-catenin activation, WNT-enriched tumors accounted for 40% to 60% of patients in our study leveraging two independent cohorts. Of note, this molecular scenario was associated with an immunological repertoire potentially denoting lower immune responsivity. For instance, in lung cancer, we have extensively documented the association between an immune excluded phenotype and decreased efficacy of ICIs (Scalera et al., 2023; Marinelli et al., 2020; Scalera et al., 2021). The same considerations extend to other treatment options (e.g., locoregional treatments). In these contexts, our model might help to better frame the population at higher risk of disease recurrence, and then candidate to treatment intensification.

5. Conclusions

Here, we described a transcriptional signature denoting hyperactivation of Wnt/β-catenin signaling that is associated with lower survival and an immune-excluded microenvironment in HCC patients. The connection between the WNT signature and survival outcomes was consistent between two independent cohorts, thus indicating the potential of the model in predicting survival outcomes. Prospective validation is needed to provide formal evidence on the connection between aberrant Wnt/β-catenin activity, *CTNNB1* mutations, and survival outcomes in HCC patients.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexmp.2026.105049.

Availability of data and materials

The RNA-seq and DNA-seq datasets generated from the Rome cohort and analyzed during the current study are available in the GEO repository series GSE270897 (temporary link with reviewer access token "ktazyiwclpethwp": https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE270897) and the EGA archive (Study ID EGAS50000000862) respectively.

Data from the TCGA HCC cohort are available at www.cbioportal.org. Immunogenomic features related to the TCGA HCC study were downloaded from the CRI iAtlas Portal (available at www.cri-iatlas.org).

CRediT authorship contribution statement

Stefano Scalera: Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Laura Cipriani:** Visualization, Methodology, Investigation, Formal analysis, Data curation. **Andrea Scarinci:** Visualization, Resources, Investigation. **Giulia Schiavoni:** Visualization, Investigation, Formal analysis. **Maurizio Fanciulli:** Visualization, Data curation. **Ludovica Ciuffreda:** Visualization, Methodology, Data curation. **Francesca De Nicola:** Visualization, Methodology, Data curation. **Frauke Goeman:** Visualization, Methodology, Data curation. **Edoardo Pescarmona:** Visualization, Resources. **Mariagrazia Diodoro:** Visualization, Resources, Methodology, Investigation. **Elisa Melucci:** Visualization, Resources, Methodology, Investigation. **Enzo Gallo:** Visualization, Resources, Methodology,

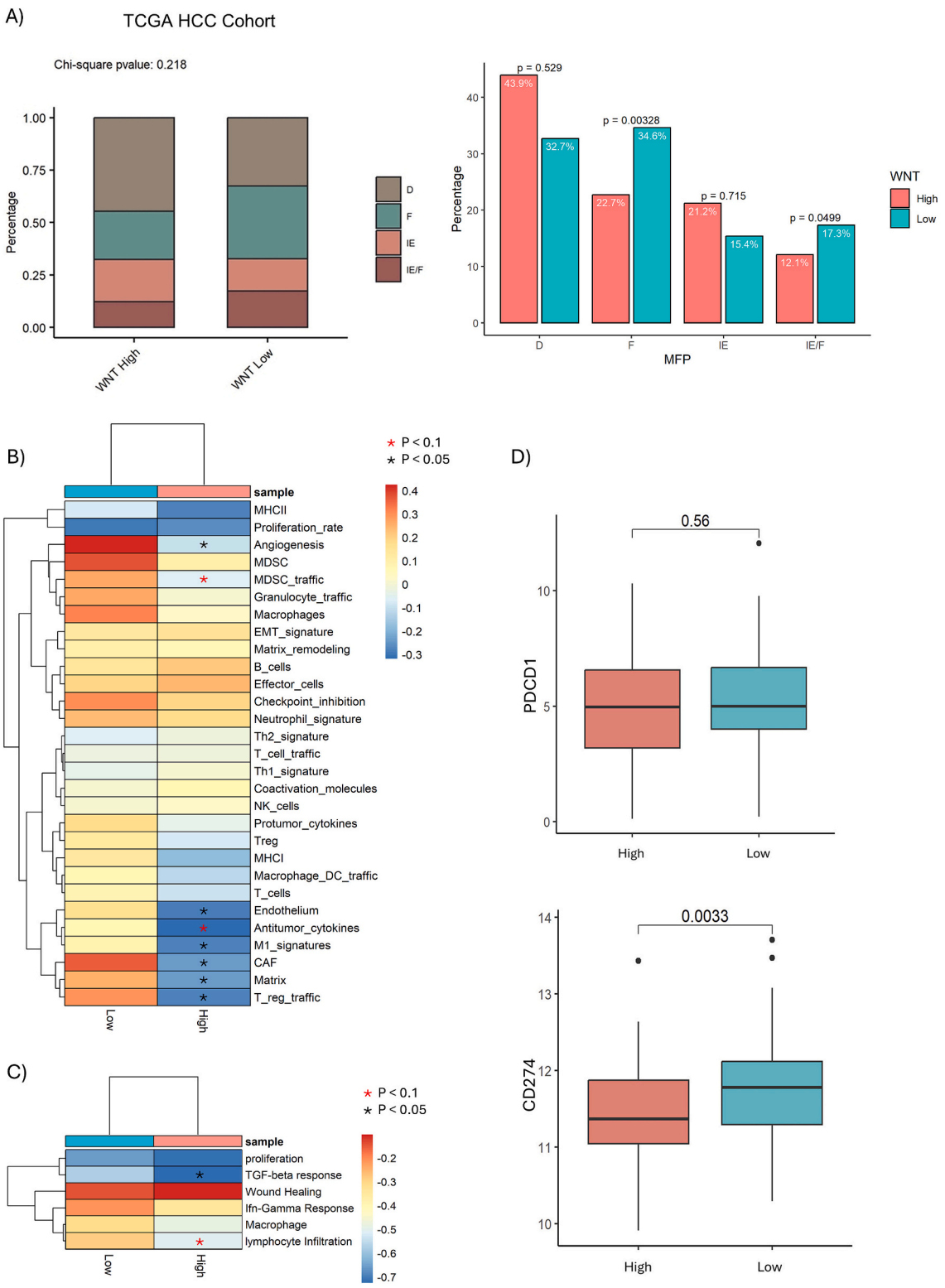

Fig. 7. Immune subtyping in the TCGA HCC study. A. Stacked bar chart showing the distribution of TIME subtypes in the WNT high and WNT low subgroups (left panel); bar chart illustrating the distribution of immunogenic subtypes across WNT high and WNT low tumors. Statistical comparisons were performed using the chi-square test. (right panel). **B.** Heatmap illustrating the 29 BostonGene TIME-related signatures used for deriving the pan-cancer TIME-based molecular subtyping. **C.** Heatmap of CRI iAtlas core immune-related signatures in the two investigated subgroups. Red asterisks denote $p < 0.1$ and black asterisks denote $p < 0.05$. **D.** Box plots for PD-1 (*PDCD1*) and PD-L1 (*CD274*) gene expression in the WNT high and WNT low subgroups (PD1, $p = 0.56$; PD-L1, $p = 0.0033$).
Abbreviations: CAF, cancer-associated fibroblast; D, immune-depleted; EMT, epithelial-mesenchymal transition; F, fibrotic; HCC, hepatocellular carcinoma; IE, immune-enriched, non-fibrotic; IE/F, immune-enriched, fibrotic; IFN-gamma, interferon gamma; MDSC, Myeloid-derived suppressor cells; MHC, major histocompatibility complex; NK, natural killer; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand; TGF-b, transforming growth factor-beta; TIME, tumor immune microenvironment; WT, wild-type. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Investigation. **Patrizia Vici:** Supervision, Conceptualization. **Laura Pizzuti:** Visualization, Methodology. **Maddalena Barba:** Visualization, Methodology. **Eriseld Krasniqi:** Visualization, Investigation. **Emanuela Dell'Aquila:** Visualization, Resources. **Chiara Manai:** Visualization, Resources. **Giancarlo Paoletti:** Visualization, Resources. **Federico Cappuzzo:** Visualization, Supervision, Resources. **Massimo Zeuli:** Visualization, Supervision. **Gennaro Ciliberto:** Visualization, Supervision. **Pasquale Perri:** Visualization, Supervision, Resources. **Gian Luca Grazi:** Visualization, Supervision, Resources. **Giulia Bon:** Writing – review & editing, Writing – original draft, Visualization. **Marcello Maugeri-Saccà:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Investigation, Funding acquisition, Conceptualization.

Consent for publication

All authors agree to the content of the paper.

Ethics approval and consent to participate

The study involving the Rome cohort was approved by the ethics committee "Comitato Etico Territoriale Lazio Area 5" based in IFO IRCCS. Written informed consent was obtained from all participants.

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Declaration of competing interest

The authors declare that they have no competing interests.

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Data availability

The RNA- and DNA-seq datasets used this study are available in the GEO repository series GSE270897 (reviewer access token "ktazyiwclpethwp") and the EGA archive (Study ID EGAS50000000862).

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