

Original research

Inflamed and non-inflamed classes of HCC: a revised immunogenomic classification

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ABSTRACT

Objective We previously reported a characterisation of the hepatocellular carcinoma (HCC) immune contexture and described an immune-specific class. We now aim to further delineate the immunogenomic classification of HCC to incorporate features that explain responses/resistance to immunotherapy.

Design We performed RNA and whole-exome sequencing, T-cell receptor (TCR)-sequencing, multiplex immunofluorescence and immunohistochemistry in a novel cohort of 240 HCC patients and validated our results in other cohorts comprising 660 patients.

Results Our integrative analysis led to define: (1) the inflamed class of HCC (37%), which includes the previously reported immune subclass (22%) and a new immune-like subclass (15%) with high interferon signalling, cytolytic activity, expression of immune-effector cytokines and a more diverse T-cell repertoire. A 20-gene signature was able to capture ~90% of these tumours and is associated with response to immunotherapy. Proteins identified in liquid biopsies recapitulated the inflamed class with an area under the ROC curve (AUC) of 0.91; (2) The intermediate class, enriched in *TP53* mutations (49% vs 29%, $p=0.035$), and chromosomal losses involving immune-related genes and; (3) the excluded class, enriched in *CTNNB1* mutations (93% vs 27%, $p<0.001$) and *PTK2* overexpression due to gene amplification and promoter hypomethylation. *CTNNB1* mutations outside the excluded class led to weak activation of the Wnt- β catenin pathway or occurred in HCCs dominated by high interferon signalling and type I antigen presenting genes.

Conclusion We have characterised the immunogenomic contexture of HCC and defined inflamed and non-inflamed tumours. Two distinct *CTNNB1* patterns associated with a differential role in immune evasion are described. These features may help predict immune response in HCC.

INTRODUCTION

Liver cancer incidence is increasing worldwide with more than 1 million annual cases expected by year 2025.^{1,2} Hepatocellular carcinoma (HCC) accounts for 90% of all primary liver cancers and is mainly

Significance of this study

What is already known on this subject?

► We previously reported the immune class of hepatocellular carcinoma (HCC), present in ~25% of patients. However, the immune traits of the remaining ~75% of HCCs are ill defined. Further, the association of response (15%–20% of HCC cases) or resistance to immune checkpoint inhibitors and the role *CTNNB1* mutations is unclear.

What are the new findings?

► By using an integrative genomic approach, we have now refined the Inflamed class (~35% of cases), which includes the immune subclass and the newly described immune-like subclass. A 20-gene signature captures this class and is associated with response to immunotherapy. Also, we characterise non-inflamed profiles and classes and decipher the potential dual role of *CTNNB1* mutations with response and evasion.

How might it impact on clinical practice in the foreseeable future?

► This revised immunogenomic classification of HCC unveils several novel mechanisms of immune response and evasion and may help to better predict the distinct patterns of outcome associated with immunotherapy in HCC. A 20-gene signature capturing the inflamed class can be tested as a direct biomarker of response.

caused by chronic hepatitis B virus (HBV), hepatitis C virus (HCV) infection, alcohol abuse and non-alcoholic steatohepatitis.^{1,2}

It is estimated that around 50%–60% of HCC patients will receive systemic treatments,³ where the combination of the immune checkpoint inhibitor (ICI) atezolizumab with bevacizumab (anti-vascular endothelial growth factor - VEGF) has recently become the new first-line treatment of advanced

HCC.^{4,5} This combination has triggered a breakthrough in HCC management,⁶ although only one-third of patients clearly respond. Thus, a refined understanding of the immune landscape of HCC to predict outcomes after ICI therapy is still lacking and there is an unmet need to define the factors determining tumour immunogenicity. Evidence in other cancer types suggests that an inherently inflamed tumour microenvironment (TME) can be leveraged by ICI therapy to elicit better outcomes, whereas immune excluded tumours are prone to resistance.⁷

We previously described the Immune class of HCC (~25% of patients), characterised by a high immune infiltration and molecular features resembling melanoma patients who respond to ICIs.⁸ In that report, we suggested that further studies should refine the immune traits of the remaining ~75% of HCC cases. Moreover, recent findings have suggested that mutations in *CTNNB1* (β catenin) and subsequent activation of the Wnt- β catenin pathway could be implicated in driving an excluded phenotype,⁹⁻¹¹ although discordant results on its predictive potential in HCC suggest the need for further analysis.¹²⁻¹⁴

In the current integrative analysis using 240 newly collected HCC samples and cutting-edge genomic technology, we define the inflamed class of HCC in ~35% of cases, including the previously reported immune subclass (22%) and a newly identified immune-like subclass (15%). In addition, we describe the non-inflamed classes which we characterise as intermediate and excluded classes with distinct molecular and immune traits. Finally, we decipher the impact of *CTNNB1* mutations in HCC and establish that while it is associated with immune exclusion in most cases, in some instances Wnt- β catenin activated tumours harbour strong interferon signalling leading to an inflamed microenvironment.

Overall, our findings hold great potential to guide the discovery of clinically useful biomarkers and lay the groundwork for the development of new combination-based therapeutic strategies.

MATERIALS AND METHODS

Patients and samples

For the purpose of this study, we collected a total of 240 clinically annotated matched tumour and non-tumoural liver samples from HCC patients undergoing resection at several institutions.¹⁵ Samples encompassed the most common etiologies (90 HCV-infected, 75 HBV-infected and 75 non-viral) and were collected on approval of the Institutional Review Board at each Institution. A second cohort of 71 tissue samples with matched baseline blood samples was used for the liquid biopsy-based biomarker analysis.¹⁶ Additional cohorts were used to validate our findings, including the The Cancer Genome Atlas Liver Hepatocellular Carcinoma (TCGA-LIHC) dataset¹⁷ and the Heptronic cohort¹⁸ (online supplemental data). **Figure 1** describes the flow chart of the study.

Methodological details on histological assessment, immunohistochemical analyses, multiplex immunophenotyping, TCR sequencing, RNA sequencing, whole exome sequencing and statistical analysis are described in online supplemental data file.

RESULTS

We first characterised the distinct immune-related classes of HCC in a new cohort of 240 HCC samples. The main clinicopathological traits of this cohort are depicted in online supplemental table 1); with a median follow-up of 55.1 months, median overall survival (mOS) was 70 months and median recurrence-free survival (mRFS) was 26.9 months (online supplemental figure 1A,B).

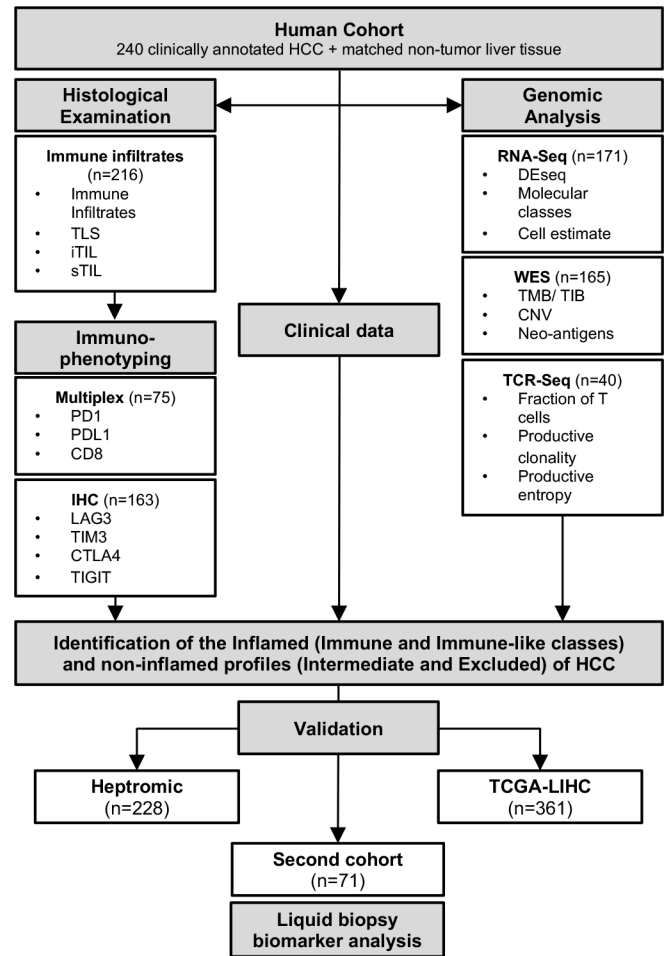


Figure 1 Flow chart of the study. A total of 240 clinically annotated HCC and matched non-tumour tissue samples were used in this study as the discovery cohort. findings were then validated in two additional datasets comprising 589 additional patients and a new cohort of 71 patients with baseline tissue and blood samples. CD8, cluster of differentiation 8; CTLA4, cytotoxic T-lymphocyte associated protein 4; HCC, hepatocellular carcinoma; iTIL, intratumoural TILs; sTIL, stromal tumour infiltrating lymphocytes; IHC, immunohistochemistry; LAG3, lymphocyte-activation gene 3; PD1, programmed cell death protein 1; PDL1, programmed death-ligand 1; TCGA-LIHC, The Cancer Genome Atlas Liver Hepatocellular Carcinoma; TIGIT, T cell immunoreceptor with Ig and ITIM domains; TIM3, T-cell immunoglobulin domain and mucin domain 3; TMB, tumour mutational burden.

Defining inflamed and non-inflamed HCCs

We first validated the immune class of HCC (22%) and its two components, the immune active and exhausted subclasses, in line with our previous findings.⁸ The immune active type presented a significantly better survival when compared with the rest of the cohort in both univariate and multivariate analysis (mOS 95 vs 51 months, $p=0.01$, (online supplemental figure 1, (online supplemental table 2, while the immune exhausted subclass predicted worse RFS (HR 2.84, 95% CI 1.22 to 6.64, $p=0.016$) (online supplemental table 2). Then, we aimed to characterise the remaining non-immune samples (78%). Among those, we depicted a subset of patients that presented high interferon signalling despite not being captured by the immune class signature (online supplemental figure 2A). Based on the expression of interferon genes, we clustered these samples into a separate class which we referred to as immune-like subclass (15%) (figure 2,

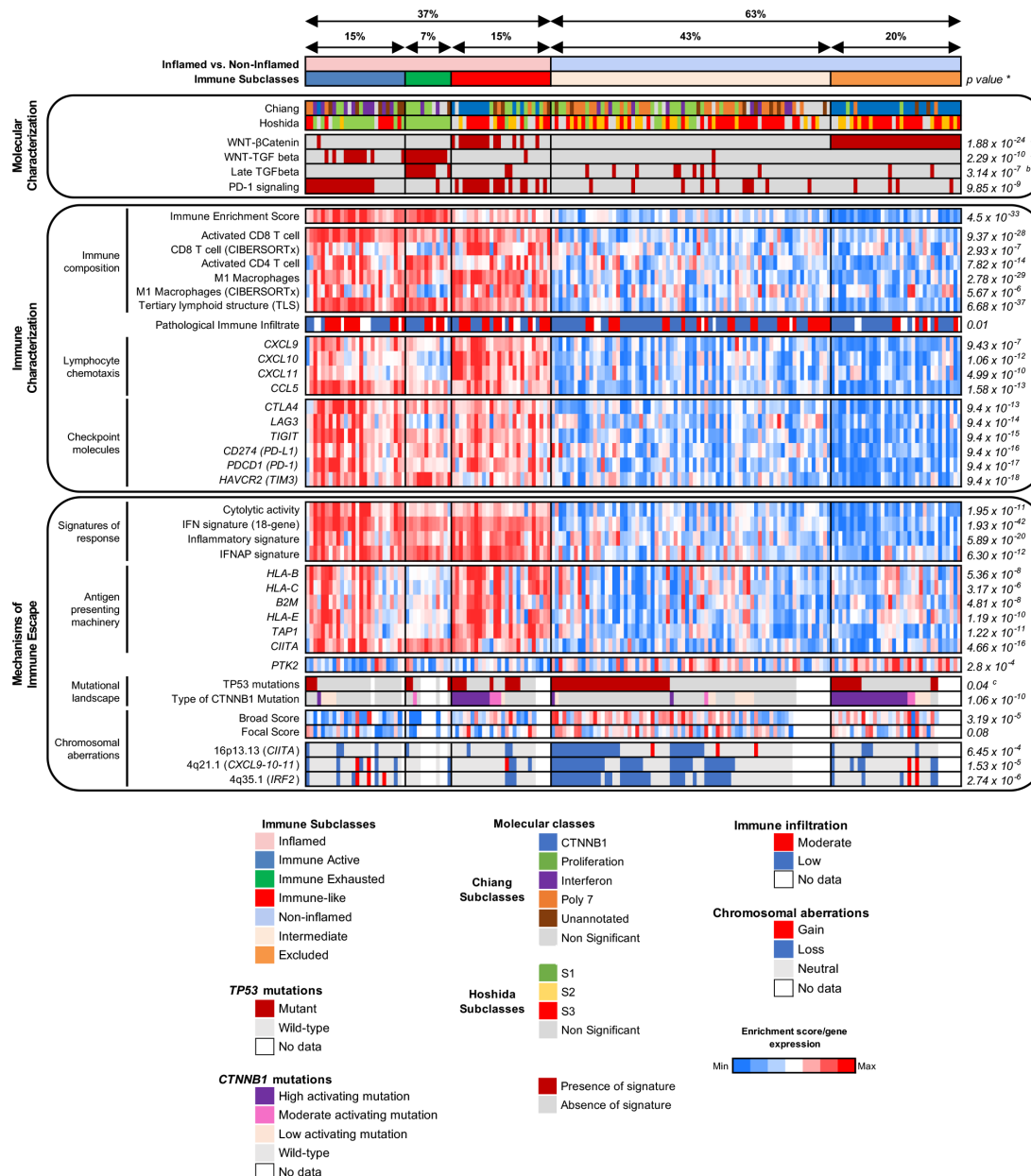


Figure 2 Heatmap representation of the main molecular and immune features of the distinct immune-related profiles. *P values shown are calculated by Student's t-test for continuous variables or Fisher's exact test for categorical variables. Unless otherwise indicated, it represents differences between the inflamed and non-inflamed classes; ^aCompares excluded versus rest of the cohort. ^bCompares exhausted versus rest of the cohort; ^cCompares intermediate versus rest of the cohort.

online supplemental figure 2A). Due to the commonalities in the immune traits between the immune and immune-like classes, including 37% of the cohort, we named these cases as inflamed class (figure 2). The remaining 63% of HCC cases depicted non-inflamed profiles. Among those, a subset of tumours showed low immune infiltration associated with the CTNNB1 class, consistent with our previous description.⁸ Using a Wnt-βcatenin signature that captures activation due to CTNNB1 mutations or other mutations in the pathway^{19 20} we were able to identify this group of tumours (20%) and are referred to as the *Excluded class* (figure 2 and online supplemental figure 2B, online supplemental table 3). The remaining non-inflamed patients were grouped in a separate class termed intermediate (43%) (figure 2). We then focused on characterising the driver traits that substantiate the uniqueness of each identified class.

Inflamed class

The inflamed class has three components: our previously described immune active and immune exhausted subclasses along with a newly identified immune-like subclass, which has been further characterised (figure 2). We were able to design a 20-gene signature that captured ~90% of cases of the inflamed class and identify blood-based biomarkers recapitulating this class.

Immune-like subclass of HCC

Tumours from patients with the immune-like subclass harboured high interferon signalling, a higher immune enrichment score ($p=6.59 \times 10^{-14}$), an enrichment in signatures capturing CD8 T cells ($p=1.26 \times 10^{-15}$), M1 macrophages ($p=2.09 \times 10^{-27}$) and

tertiary lymphoid structures ($p=3.09\times 10^{-20}$, figure 2) when compared with the non-inflamed profiles. We observed that the immune-like subclass presented a higher enrichment in PD-1 signalling pathway (62% vs 13%, $p=1.25\times 10^{-6}$) and expression of checkpoint molecules including *CTLA4*, *PD-1* and *PD-L1* ($p<1\times 10^{-3}$, figure 2). Furthermore, there was a significant enrichment in signatures involved in response to immunotherapy, such as interferon signalling, the HCC IFNAP signature, cytolytic activity and the HCC inflammatory signature ($p<1\times 10^{-3}$, figure 2). Of note, the immune features of this class, including high expression of checkpoint molecules, inflammation-related pathways and enrichment in signatures of response to immunotherapy, were similar to those of the immune subclass⁸ (figure 2). The immune-like subclass patients did not have differences in survival when compared with the non-inflamed profiles—as opposed to the immune active type (online supplemental figure 1)—or any clinical or pathological differences when compared with the other immune types (online supplemental table 4).

We identified striking molecular differences,^{19 21} however, between the immune and immune-like subclasses. The immune-like was enriched in Hoshida S2 (19% vs 3%, $p=0.04$) and Chiang CTNNB1 classes (46% vs 3%, $p=2.87\times 10^{-5}$) and presented a significant exclusion of the Hoshida S1 (8% vs 68%, $p=1.6\times 10^{-6}$) and Chiang IFN class (0 vs 32%, $p=9.17\times 10^{-4}$) when compared with the immune subclass (figure 2, online supplemental tables 5 and 6). Also, we observed an enrichment in the liver-related Wnt- β catenin signalling pathway (54% vs 3%, $p=2.35\times 10^{-6}$) and a significant exclusion of Wnt-TGF β signalling (4% vs 55%, $p=1.11\times 10^{-5}$) in the immune-like subclass compared with the immune (figure 2). Altogether, these data suggest that our newly identified immune-like subclass captures a subset of tumours with similar immune features but distinct molecular traits compared with the other immune types.

Characterisation of the inflamed class immunophenotype

We next explored the immune landscape of the inflamed class by performing transcriptomic deconvolution, TCR sequencing, multiplex immunophenotyping, histological assessment and IHC. Histologically, patients in the inflamed class showed a higher immune infiltration when compared with the non-inflamed profiles (53% vs 32%, $p=0.01$, figure 3A, online supplemental table 7), higher presence of stromal tumour infiltrating lymphocytes (sTILs, 42% vs 18%, $p=0.01$), intratumoural TILs (iTILs, 33 vs 16%, $p=0.07$) and tertiary lymphoid structure (high TLS density, 27% vs 12%, $p=0.09$, figure 3B–D, online supplemental figures 3 and 4, online supplemental table 8).

We additionally performed IHC for CTLA4, LAG3, TIGIT and TIM3. The inflamed class presented a significant enrichment for LAG3 (39% vs 19%, $p=0.049$) while the immune class presented a higher presence of CTLA4 (58% vs 29%, $p=0.03$, online supplemental figure 4) when compared with the non-inflamed profiles. No differences were observed among the immune-related subclasses for TIM3 and TIGIT.

To further assess the TME, we performed multiplex immunophenotyping of CD8, PD-1 and PD-L1 in a subset of tumours. Consistent with our transcriptomic assessment, we observed an enrichment of intratumoural CD8 T cells (CD8 $\geq 1\%$, 58% vs 30%, $p=0.08$) and PD-L1 (PD-L1 $\geq 1\%$, 21 vs 4%, $p=0.19$) in the inflamed profiles when compared with the non-inflamed (online supplemental figures 3 and 4).

Next, we applied CIBERSORTx to characterise the immune microenvironment. We observed a significantly higher fraction of CD8 T cells ($p=3.51\times 10^{-7}$) and M1 macrophages ($p=1.82\times 10^{-4}$)

in the inflamed class when compared with the non-inflamed profiles and a significant exclusion of M2 macrophages in the immune-like subclass ($p=1.78\times 10^{-6}$, figure 3E). We further validated these findings by applying xCell deconvolution (online supplemental figure 5).

Subsequently, we performed TCR sequencing to better characterise the TCR repertoire. Consistent with our previous analysis, the inflamed class contained a higher fraction of T cells, a higher number of productive rearrangements and a richer and more diverse T cell repertoire when compared with the non-inflamed profiles (figure 3F–G, online supplemental figure 6). Overall, TCR clonality was low (range 0.04–0.36) indicating that HCC samples at baseline are mostly polyclonal. Interestingly, cases belonging to the immune-like subclass showed a higher productive clonality ($p=0.01$) when compared with the other classes, indicating a probable oligoclonal expansion of T cells (figure 3H). When we examined the clones with the highest productive frequency, no shared sequences were observed across groups and within each group, suggesting that the top clones are unique in each patient.

Overall, this suggests that *Inflamed HCC* tumours, in addition to containing a higher infiltration of T cells and CD8 T cells, display a more diverse T-cell repertoire.

Discovery of the inflamed gene signature

In order to provide a tool to accurately capture HCC cases belonging to the inflamed class, we designed and validated a 20-gene signature that we refer to as ‘inflamed signature’ (online supplemental table 9). The most characteristic gene components of the *Inflamed signature* were related to T-cell signalling and activation (*CD3D*, *CD2*, *LCP2*), lymphocyte chemotaxis (*CXCL9*, *CCL5*) and cytolytic activity (*GZMA*, *GZMB*) (online supplemental figure 7A). Similarly, they were enriched in pathways related to antigen presentation, T cell signalling and B cell activation (online supplemental figure 7B). In the discovery cohort of 240 cases, the signature had an accuracy of 89%, sensitivity and specificity of 80% and 95% and a positive and negative predictive value of 89% and 91%, respectively (online supplemental figure 7C), (online supplemental table 10). Increasing the number of genes in the signature did not increase its accuracy (online supplemental figure 7D).

We validated our inflamed signature in the Heptronic⁸ and the TCGA-LIHC cohorts.²² In both cohorts, the signature had an accuracy of $\sim 90\%$ (online supplemental figure 7C) and online supplemental table 10). Additionally, we observed that in both validation cohorts, the inflamed class showed similar molecular traits to our discovery cohort, with increased interferon signalling, higher immune infiltration and higher cytolytic activity, among other features (online supplemental figure 8). When tested in an external cohort of advanced HCC patients treated with ICIs,²³ patients who responded showed a significant overexpression of the inflamed signature when compared with non-responders ($p=0.047$, online supplemental figure 9), suggesting it could accurately predict response to ICIs.

A liquid-biopsy based biomarker recapitulating the inflamed class

To identify patients belonging to the *Inflamed class* by using liquid biopsy-based biomarkers, we assembled a new cohort of 71 HCC samples with matched tumour tissue, non-tumour tissue and baseline blood samples in 68 cases (online supplemental figure 10). We measured 92 protein biomarkers in peripheral blood and built a predictive model to recapitulate the *Inflamed class*. This allowed us to devise a 13-protein signature that captured the inflamed class with an AUC of 0.91, an accuracy of 85% and a sensitivity of 92% (figure 3I,J).

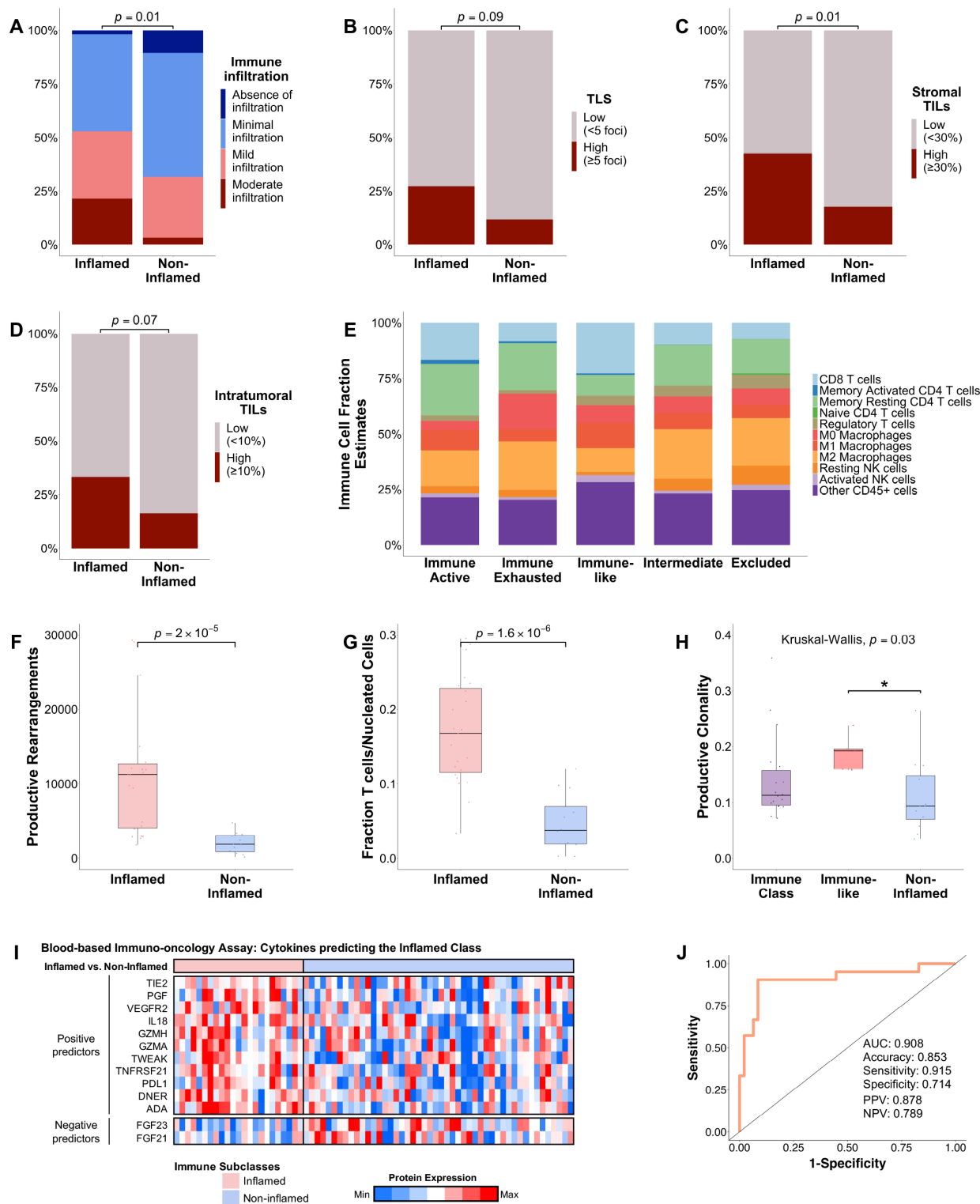


Figure 3 The inflamed class shows high immune infiltration and features of inflammation. (A–D) Barplot depicting (A) the richness of the immune infiltrate, (B) density of TLS, (C) the stromal TILs and (D) the intratumoural TILs as assessed by H&E examination. (E) Stacked barplot depicting the fraction of 22 immune cell types inferred by CIBERSORTx. (F–H) TCR sequencing results showing (F) the number of productive rearrangements, (G) fraction of T cells and the (H) productive clonality. (I) Heatmap representation of 11 cytokines which positively predict the inflamed class and two cytokines which were enriched in the non-inflamed class. (J) AUC showing the performance of the devised 13-protein signature in capturing the *Inflamed class*. P value are calculated by (A–D) Fisher’s exact test, (F–G) Wilcoxon’s rank-sum test and (H) Kruskal-Wallis test with post hoc Dunn’s test. * $P < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. AUC, area under the ROC curve; NPV, negative predictive value; PPV, positive predictive value; TILs, tumour infiltrating lymphocytes; TLS, tertiary lymphoid structures.

Non-inflamed classes

Overall, the non-inflamed classes (~63% of cases) depict immune features that are significantly distinct from the

inflamed class (figure 2). Nonetheless, the non-inflamed cases were also heterogeneous in immune and molecular characteristics, allowing us to differentiate two distinct classes based on

the underlying mechanisms of immune evasion: the intermediate and the excluded classes.

The intermediate class

In terms of immune traits, the intermediate class presented a mild infiltration of immune cells as assessed by the ESTIMATE and xCell tools. Immune infiltration was significantly decreased when compared with the inflamed class ($p=9.31 \times 10^{-24}$) but significantly higher than in the excluded class ($p=5.46 \times 10^{-7}$) (figure 2).

In terms of somatic mutations, tumours belonging to the intermediate class were enriched in *TP53* mutations when compared

with the inflamed class (49% vs 29%, $p=0.04$) and the rest of the cohort (49% vs 31%, $p=0.04$, (figure 2, online supplemental figure 11). We next explored the status of chromosomal aberrations, which have been associated with immune evasion,^{24 25} by using the CNApp algorithm²⁶ to infer copy-number scores and genomic imbalances. We first calculated a single-sample score for broad and focal events, which accounts for the number, amplitude and length of the broad and focal segments, respectively. Interestingly, there was a significant increase in broad and focal scores in both the intermediate and excluded classes (figure 4A,B). To further validate these findings, we applied the GISTIC algorithm, revealing an increased number of focal

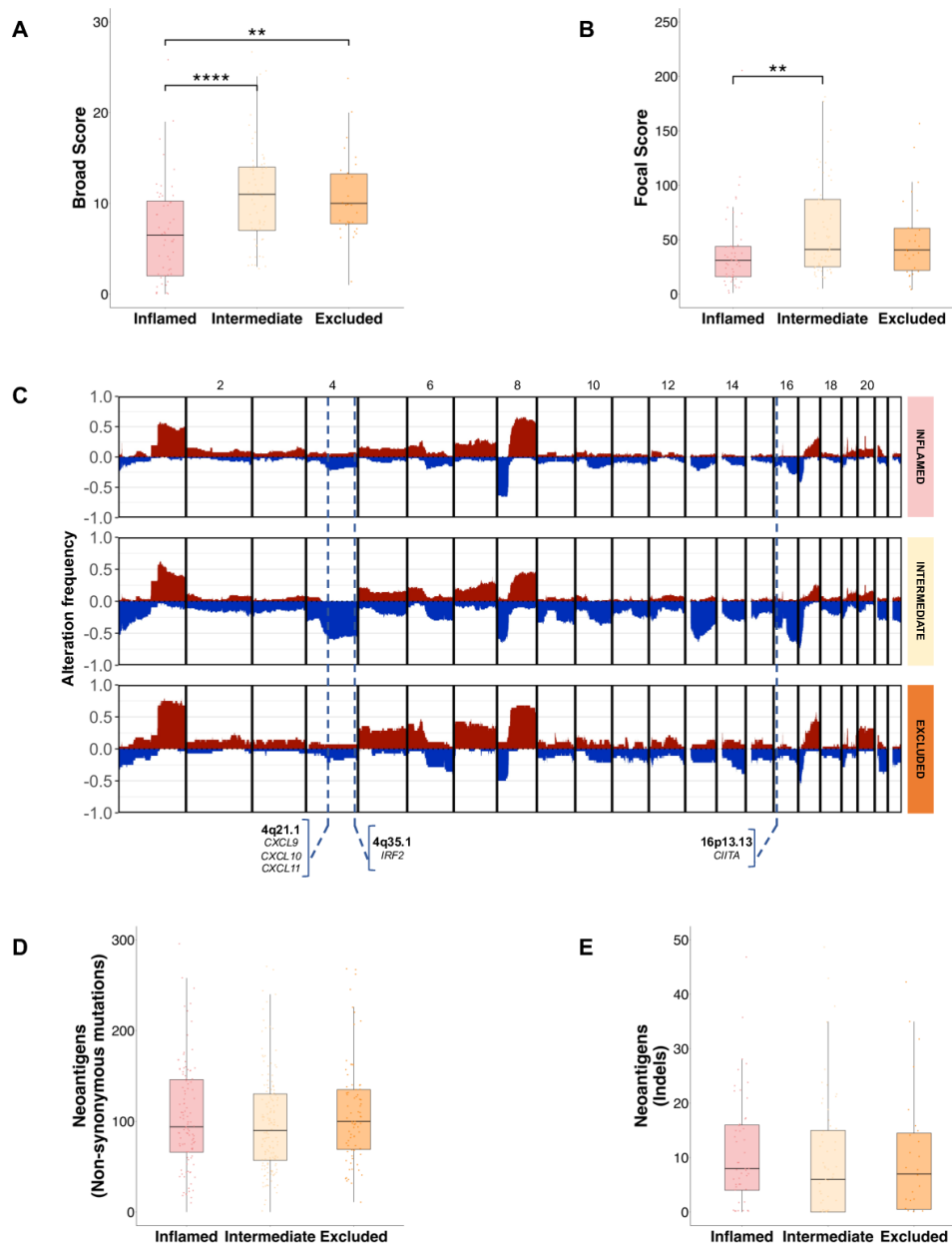


Figure 4 Genomic overview of the distinct immune classes of HCC. (A, B) Boxplot depicting the distribution of (A) broad and (B) focal chromosomal aberration as assessed by the CNApp algorithm. Kruskal-Wallis, $p=9.5 \times 10^{-5}$ and Kruskal-Wallis, $p=0.02$, respectively. (C) Genomic overview showing the percentage of samples with copy number events among each immune class. Blue represent deletions, red represent gains, the Y axis depicts the percentage of these events across each immune class. Potentially Impactful subcytobands in immune evasion are indicated with a dotted line. (D) Boxplot showing the distribution of inferred neoantigens and (E) neoantigens from insertions and deletions. Kruskal-Wallis, $p=0.87$ and Kruskal-Wallis, $p=0.3$, respectively. For (A, B, D, E), p values are calculated by Kruskal-Wallis test with post hoc Dunn's test. * $P < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. HCC, hepatocellular carcinoma.

deletions in the intermediate and excluded classes compared with the Inflamed class and an increased burden of broad deletions in the intermediate class (online supplemental figure 12). There was a significantly increased number of losses in genomic regions that harboured genes involved in antigen presentation or interferon signalling in the intermediate class compared with the rest of the cohort, including subcytobands 16p13.13 (harbouring *CIITA*^{27,28}, 43% vs 16%, $p=6.45 \times 10^{-4}$), 4q21.1 (harbouring *CXCL9*, *CXCL10*, *CXCL11*, 54% vs 19%, $p=1.53 \times 10^{-5}$) and 4q35.1 (harbouring *IRF2*,^{29,30} 56% vs 18%, $p=2.74 \times 10^{-6}$) (figure 4C, (online supplemental figure 13), (online supplemental tables 11 and 13). These deletions also had an impact on the gene mRNA expression (online supplemental figure 14).

Finally, we explored the tumour mutational burden (TMB) and neoantigen burden, which have been associated with inflammation and immune response.^{31,32} Overall, the median number of non-synonymous mutations was 77 (range 14–226) and median TMB was 2.6 mutation/Mb (range 0.2–8.2), with no differences among classes. A median of 35 (range 4–164) immunogenic mutations (defined as those mutations that were predicted to generate at least one HLA-binding epitope; ~45% of the total mutations) were identified, with no differences among classes. The median number of expressed neoantigens and high-affinity neoantigens (defined as those with a rank binding affinity <0.5%) across the whole cohort was 99 (range 4–456) and 27 (range 1–124), respectively. No differences were found among all classes in this discovery cohort (figure 4D, (online supplemental figure 15A,B) and in TCGA-LIHC³³ (online supplemental figure 15C). Also, we found no differences among classes in the distribution of neoantigens derived from indels in our cohort (figure 4E, (online supplemental figure 15D,E) and TCGA-LIHC³³ (online supplemental figure 15F).

Altogether, these data show that the intermediate class is characterised by an enrichment in *TP53* mutations and higher levels of chromosomal instability, with frequent deletions in genes related to antigen presentation or interferon signalling. These structural features are associated with a significant decrease in immune infiltration and immune score observed in the intermediate class (figure 2).

The excluded class

The excluded class (20% $n=34/171$) presented the lowest immune infiltration ($p=2.82 \times 10^{-10}$) (figure 2) and was characterised by a significant enrichment of *CTNNB1* mutations when compared with the *Intermediate class* (93 vs 17%, $p=5.55 \times 10^{-12}$) and the rest of the cohort (93 vs 27%, $p=1.06 \times 10^{-10}$, figure 5A). As different *CTNNB1* genotypes result in different levels of activation of the Wnt- β -catenin pathway,³⁴ we further analysed the type of *CTNNB1* mutations in our cohort. Most *CTNNB1* mutations were located on exon 3 (91.2%) (online supplemental figure 16A). We classified these mutations in high, moderate and low activation according to a previously established classification³⁴ (online supplemental figure 16B) and observed that 88% of mutations in the excluded class caused high or moderate activation of the Wnt- β -catenin pathway (figure 5A,B). In addition, we also observed a higher presence of missense variants in *APC* compared with the intermediate class (11% vs 0%, $p=0.03$), which also activates the Wnt- β -catenin pathway. Conversely, no differences were observed with *AXIN1* variants (3.6% vs 13%, $p=0.27$), which is consistent with previously published data showing a lack of activation of the Wnt- β -catenin pathway as a result of these mutations.^{35,36} We further characterised the excluded class and observed a significant overexpression of

PTK2 when compared with the intermediate ($p=0.02$) and the inflamed class ($p=1.13 \times 10^{-5}$) which was validated by IHC (online supplemental figure 17A-D). To understand the mechanisms underlying *PTK2* overexpression, we identified those samples that had an expression value of the gene that was at least twofold times higher than the mean expression in non-tumoural tissue. According to this definition, 32% of samples in our cohort overexpressed *PTK2*, which was similar to the one in TCGA-LIHC (27%). We found that 70% of overexpressing samples harboured a gene-amplification (>3 copies of the 8q24.3 subcytoband) (online supplemental figure 17E), which increased to 83.5% in TCGA-LIHC. When incorporating promoter region hypomethylation of *PTK2* in TCGA-LIHC, almost 95% of the samples had an identifiable mechanism of overexpression (online supplemental figure 17F). This suggests that both gene amplification and promoter region methylation are major drivers of *PTK2* overexpression in HCC.

Overall, the excluded class is characterised by the presence of highly activating *CTNNB1* mutations and overexpression of *PTK2*.

CTNNB1 mutations are associated with two distinct types of immune activation

Moderate or high-activating *CTNNB1* mutations in the intermediate and immune class were significantly less prevalent compared with the excluded class (88% vs 27%, $p=5.23 \times 10^{-4}$; 88 vs 33%, $p=0.01$, respectively) (figure 5A,B). Even though most mutations in the immune subclass were low-activating mutations, 93% of mutations in the immune-like subclass were either moderate or high-activating mutations, which was no different than the excluded class (93% vs 88%, $p>0.05$) (figure 5B,C). This is consistent with our previous observation showing an enrichment of the Wnt- β -catenin activation pathway in the immune-like when compared with the immune subclass (figure 2). Therefore, we next focused on understanding the implications Wnt- β -catenin activation, which occurred in 49 of our samples (figure 6A). In two-thirds of cases Wnt activation was associated with non-inflamed classes ($n=34$, 69%)—described as a potential mechanism of immune exclusion,^{10,11} but in one-third, it was identified within the inflamed class ($n=15$, 31%). While the former group was clearly enriched in the excluded class and dominated by a paucity of immune infiltrate, the latter group was dominated by a significant enrichment of immune infiltration, activated CD8⁺ and CD4⁺ T cells, signatures of immune response, inflammation and interferon signalling (figure 6A). Differential gene expression analysis between inflamed and non-inflamed Wnt- β -catenin tumours (figure 6B, (online supplemental table 14) and subsequent pathway enrichment analysis (online supplemental figure 16C, online supplemental table 15), confirmed that most of the enriched pathways in the inflamed profiles were immune-related. Histological analysis of the immune infiltrates further confirmed these findings. We observed a higher rate of immune cell infiltration (54% vs 22%, $p=0.07$) and a higher density of TLS (≥ 5 foci, 50% vs 0%, $p=3.42 \times 10^{-3}$) in the inflamed profiles when compared with the non-inflamed (figure 6C,D). Moreover, there was a significant enrichment of sTILs (sTILs $\geq 30\%$, 63% vs 5%, $p=3.05 \times 10^{-3}$) and a trend in iTILs (iTILs $\geq 10\%$, 38% vs 5%, $p=0.06$) (online supplemental figure 16D).

Collectively, these findings suggest that two-thirds of Wnt- β -catenin activated tumours are characterised by immune paucity and exclusion, while one-third of tumours with this activation are counterbalanced by mechanisms leading to high

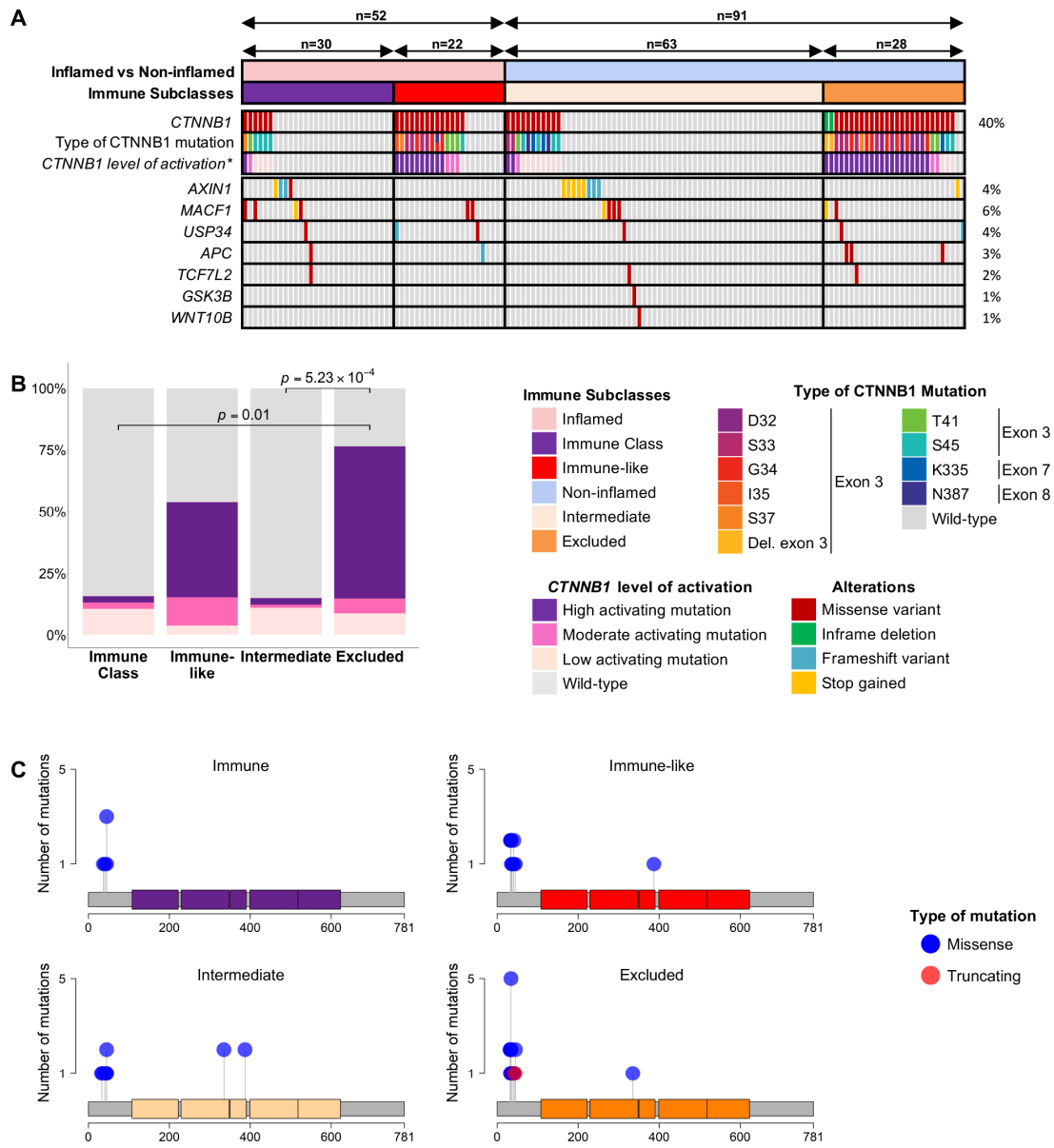


Figure 5 Mutational landscape of *CTNNB1* mutations across the immune class. (A) Heatmap representation of the distribution of mutations of key genes involved in the Wnt- β -catenin pathway. (B) Stacked barplot showing the distribution of the type of *CTNNB1* mutations across immune classes. (C) Lollipop plots showing the distribution of *CTNNB1* mutations in the distinct immune classes. P values are calculated by (B) Student's t-test and (C) Fisher's exact test. **CTNNB1* level of activation is based on Rebouissou S, Franconi A, Calderaro J, et al.³⁴

immune infiltration and inflammation. Gene set enrichment analysis revealed enhanced immune-related pathways in the inflamed profiles, such as IFN γ signalling, allograft rejection, complement activation and inflammatory response, among others (online supplemental figure 16E, online supplemental table 16). Conversely, there was no differences in the mechanism responsible for activation of the Wnt- β -catenin pathway (assessed by 19 gene signatures capturing canonical and non-canonical Wnt- β -catenin activation) (online supplemental tables 17 and 18).

Since evidence suggests that Wnt- β -catenin drives immune exclusion in HCC by interfering with dendritic cell and lymphocyte chemotaxis (through *CCL5* and *CCL4*^{10 11}) as well as NK cell chemotaxis (through NKG2D ligands³⁷), we investigated whether these mechanisms were preserved in these two distinct profiles. The inflamed profiles expressed more *CCL5*, *CCL4* and other major cytokines involved in lymphocyte chemotaxis,

such as *CXCL9*, *CXCL10* and *CXCL11* (figure 6E). Consistently, CIBERSORTx deconvolution analysis revealed higher proportions of CD8 +T cells in inflamed profiles compared with non-inflamed profiles (online supplemental figure 16F). Conversely, we did not find differences in the expression of most NKG2D ligands between the inflamed and non-inflamed profiles, including *MICA*, *ULPB1* and *ULPB2* (figure 6E), a finding that was consistent with CIBERSORTx and xCell deconvolution results that showed no differences in infiltration of activated NK cells (online supplemental figure 16G,H). These data suggest that the inflamed profiles include enhanced *CCL5* expression and T cell recruitment despite activation of the Wnt- β catenin pathway.

We further analysed the differential gene expression data and observed there was a significant upregulation of genes involved in antigen type I presentation in the inflamed profiles, including *HLA-B*, *B2M* and *TAP1*, among others. Defects in the antigen

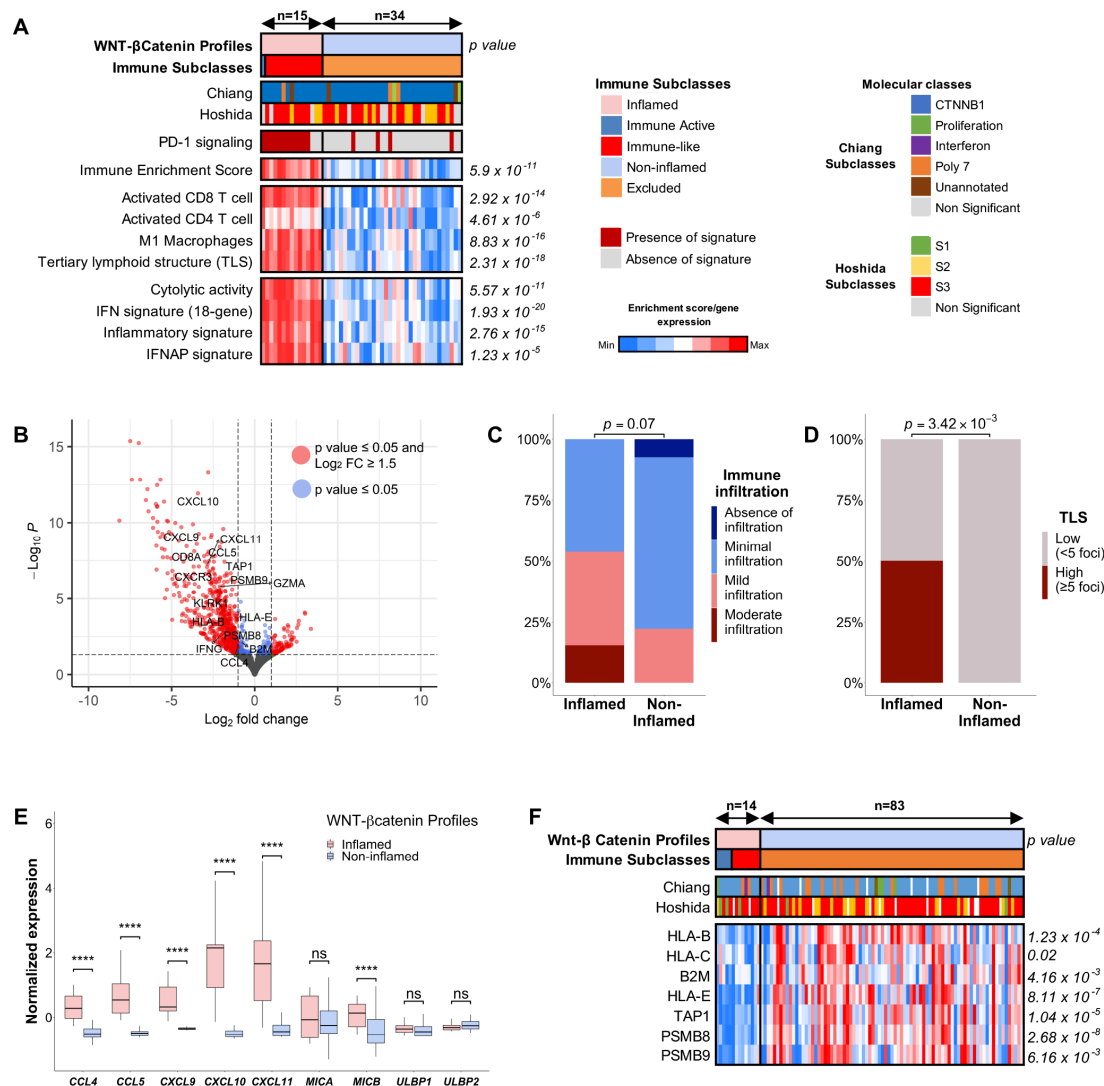


Figure 6 Two distinct profiles of Wnt-βcatenin activated tumours are identified based on immune features. (A) Heatmap representation of the main immune features of the distinct profiles. (B) Volcano plot showing the differentially expressed genes between inflamed and non-inflamed profiles. (C, D) Barplot representation of (C) the richness of the immune infiltrate and (D) TLS density as assessed by H&E examination. (E) Boxplot comparing the expression of cytokines and ligands repressed by Wnt-βcatenin pathway. (F) Heatmap comparing the methylation of genes involved in antigen type I presentation in the TCGA cohort. P values are calculated by (A, F) Student's t-test, (C, D) Fisher's exact test and (E) Wilcoxon rank-sum test. *P<0.05; **p<0.01; ***p<0.001; ****p<0.0001. ns, not significant. TCGA, The Cancer Genome Atlas Liver Hepatocellular Carcinoma; TLS, tertiary lymphoid structures.

presentation machinery, including copy-number deletions and epigenetic modifications such as hypermethylation, have been related to immune escape.³⁸ We found no enrichment in copy-number losses in antigen presenting genes. To analyse possible epigenetic changes, we explored the TCGA-LIHC cohort. There was significant hypermethylation of genes involved in antigen type I presentation including *TAP1*, *B2M*, *HLA-B* and *HLA-C* (figure 6F) in Wnt-βcatenin HCCs from the non-inflamed group, which, as expected, inversely correlated with their expression levels (online supplemental figure 18)

Overall, these data suggests that while ~70%–85% of tumours with activation of the Wnt-β catenin pathway show features of immune exclusion and immune cell paucity, ~15%–30% of tumours with activation of this pathway express features of inflammation and immune activation. Importantly, these tumours overexpress *CCL5* and have enhanced CD8+T cell infiltration. Complementary mechanisms, such as hypermethylation

of antigen-presenting genes, may contribute to these phenotypic differences.

DISCUSSION

The development of ICIs has transformed the field of immunoncology and revolutionised the management of cancer. However, only a small fraction of patients (~20%) present a durable response in HCC. Therefore, there is an ongoing unmet need to identify biomarkers that accurately predict which patients will benefit from this form of therapy. Recently, significant differences have been uncovered in the clinical outcomes following ICIs based on underlying liver disease aetiology, with a significantly increased benefit in viral-related HCC compared with non-viral.³⁹ This finding adds a new layer of complexity to the already daunting field of biomarker discovery. Thus, a thorough understanding of the HCC immune microenvironment and

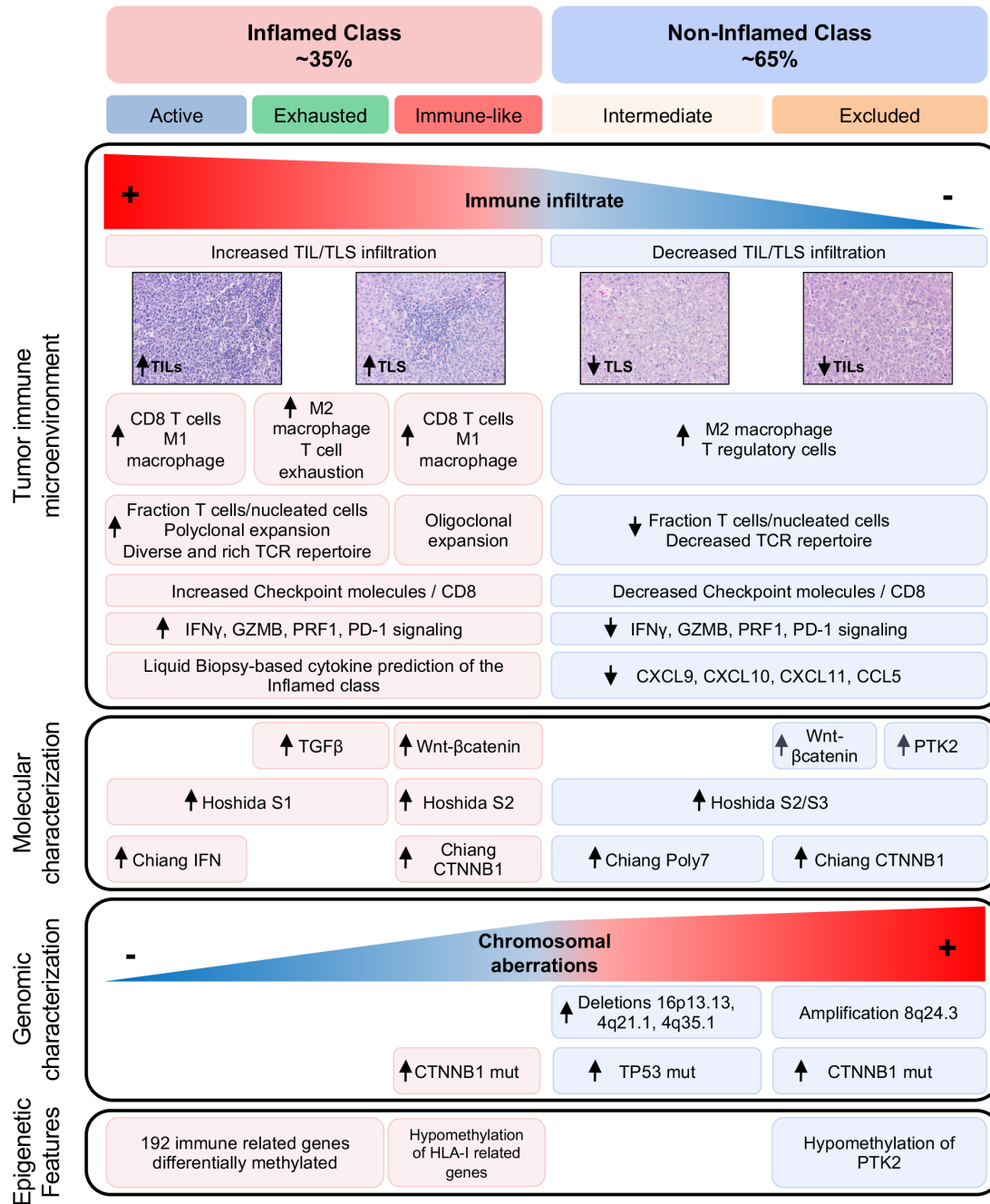


Figure 7 Graphical representation of the distinct immune profiles of HCC. The figure summarises the main molecular and histopathological features according to current findings and data previously published.^{3,8} HCC, hepatocellular carcinoma; TIL, tumour infiltrating lymphocyte.

its molecular underpinnings is imperative to guide the rational discovery of clinically useful biomarkers.

In this study, we have built on our previous findings⁸ to provide an in-depth characterisation of the HCC immunological classes (figure 7). First, we identify a cluster of tumours harbouring immune traits—the so-called immune-like subclass—resembling our previously described immune subclass⁸ but not captured by the immune signature and presenting significant activation of the Wnt-βcatenin pathway due to *CTNNB1* mutations. Overall, the proposed inflamed class accounts for ~35% of HCC cases and presents high interferon signalling, PD-1 signalling and overexpression of genes related to lymphocyte chemotaxis such as *CXCL9* and *CXCL10*. We have, therefore, developed a novel inflamed signature capable of identifying

these tumours with an accuracy of ~90% and have validated it in three additional cohorts. Additionally, we provide preliminary evidence supporting the role of liquid biopsy in identifying this class. Importantly, these inflamed HCC tumours were enriched in two recently reported inflammatory signatures^{14,40} predicting ICI response in advanced HCC patients and was enriched in responders to ICIs in a small published cohort.⁴¹ These findings strongly support the notion that inflamed HCC tumours represent potential responders to ICI therapies, a feature consistent with a report where HCCs responding to ICI after sorafenib progression present an inflamed phenotype and higher infiltrate of cytotoxic T lymphocytes.⁴² Finally, we propose a diagnostic algorithm which aims to implement this classification in clinical practice to further evaluate the predictive potential of the

Inflamed class in ICI-treated HCC cohorts (online supplemental figure 19).

Tumours immunologically classified as intermediate showed enrichment of *TP53* mutations and losses in genes related to antigen presentation and interferon signalling. Interestingly, loss of *TP53* in cancer cells promotes the recruitment of immune suppressive cells and attenuates the response of both cytotoxic and T-helper cells.^{43–44} Therefore, the increased rate of *TP53* mutations seen in the intermediate class could be key in promoting an immunosuppressive microenvironment. Furthermore, we show that these non-inflamed profiles harbour more chromosomal aberrations, with significantly more broad and focal chromosomal alterations, which in line with previous pancreatic cancer analyses.^{24–25–45} We find significantly higher deletions in genes related to interferon signalling and antigen presentation in the *Intermediate class*, which could potentially drive this non-inflamed phenotype.^{28–30–46} Functional studies are needed to validate these findings.

Compelling experimental evidence in an immunocompetent animal model of HCC has established that activation of the Wnt- β -catenin pathway may promote immune exclusion through a defective recruitment of lymphocytes and dendritic cells.¹¹ In the current study, deep immunophenotyping combining multiplex immunofluorescence and transcriptomic analysis of the non-inflamed profiles has revealed that ~20% of HCC patients present activation of Wnt- β -catenin and low immune infiltration and an overexpression of *PTK2*. We coined this group the excluded class. An intriguing finding in our study is the observation that ~15%–30% of tumours with high activation of the Wnt- β -catenin pathway belong to the inflamed class. When compared with the excluded class, these tumours express more *CCL5* and *CCL4*, two cytokines regulating lymphocyte chemotaxis that are usually repressed by the Wnt- β -catenin pathway.^{10–11} Conversely, there are no differences in the expression of the NKG2D ligands *MICA*, *ULPB1* and *ULPB2*, involved in cancer immunosurveillance and also downregulated by the Wnt- β -catenin pathway.³⁷ Furthermore, there is an upregulation of MHC-I related molecules in the inflamed Wnt- β -catenin activated profiles, accompanied by a significant demethylation of these genes.⁴⁷ Therefore, these data suggest that the immune landscape of Wnt- β -catenin activated tumours is heterogeneous and the immune phenotype could be a result of an altered balance between the immunosuppressive function of Wnt- β -catenin and the pro-inflammatory interferon pathway. Importantly, the distinction between these two profiles could partly clarify the discrepancies observed in the predictive value of *CTNNB1* mutations in HCC.^{12–14}

Overall, we have defined a new inflamed profile of HCC with high interferon signalling despite presenting an enrichment in *CTNNB1* mutations. Furthermore, we have described new potential mechanisms of immune evasion in HCC including *TP53* mutations, deletions in genes related to interferon signalling and antigen presentation and hypermethylation of genes involved in antigen type I presentation. We foresee that this classification will enable a better stratification of patients, although further studies are needed to establish its predictive value.

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Correction notice This article has been corrected since it published Online First. The author's name, Laura Torrens, has been updated.

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Contributors DS and JML designed the study. CM, FC, PH, MT-M, MP, DS and JML established and clinically annotated the cohort. CM, WQL, EH, MD and ST contributed to pathological characterisation of tumours and analysis of IHC/Multiplex immunofluorescence. MM, contributed to RNA and DNA isolation as well as to IHC profiling. CM, FC, PH, HW, AU, MP and DS contributed to data analysis. RP, LTF, AM, PT, JC, ET, EC, MS, JN, VM, JA, SR, MS, AV and SLF provided scientific input. CM, FC, DS and JML wrote the manuscript with contributions from all authors. DS and JML jointly supervised the work. JML is the author responsible for the overall content as the guarantor.

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Patient consent for publication Consent obtained directly from patient(s)

Ethics approval This study was performed including samples collected on approval of the Ethical Committee of Clinical Investigation from Hospital Clínic de Barcelona (ID: 2010-5896), IRB approval from Mount Sinai Hospital under protocol #: STUDY-11-00114 and in accordance with Mongolian regulations, the National Cancer Center and the Ministry of Health of Mongolia (ID: HS#: 15-00995). Participants gave informed consent to participate in the study before taking part.

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Data availability statement Data are available in a public, open access repository. Data are available on reasonable request. The RNAseq and Whole-exome sequencing data for the primary cohort have been deposited at the European Genome-Phenome Archive (EGA), which is hosted by the European Bioinformatics Institute (EBI) and the Centre for Genomic Regulation (CRG) (Accession code EGAS00001005364). The TCRseq and any other relevant data can be obtained from

the corresponding authors upon reasonable request. The data used for second cohort where the liquid-biopsy analysis was performed has been deposited at GEO with accession number GSE174570.

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