Title: Molecular markers of response to anti-PD1 therapy in advanced hepatocellular carcinoma

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Financial disclosures: This study was supported by a research grant from Bayer Pharmaceuticals to JM Llovet, MD.

PKH is supported by the fellowship grant of the German Research Foundation (DFG, HA 8754/1-1). FC is supported by a AECC Clínico Junior grant, ID code (CLJUN20007CAST). CAO is supported by a Fulbright fellowship. MP received a "Juan Rodés" scholarship grant from Asociación Española para el Estudio del Hígado (AEEH). J.U.M. is supported by grants from the German Research Foundation (MA 4443/2-2; SFB1292), the Volkswagen Foundation (Lichtenberg program). AV is funded by the DFG, (SFB/TRR 209 - 314905040, and Vo959/9-1). TM is funded by the NIHR UCH Biomedical Research Centre and Accelerator Award (HUNTER, Ref. C9380/A26813, partnership between the CRUK, AECC and AIRC). LRR and MAMA are supported by the Mayo Clinic Hepatobiliary SPORE (P50 CA210964). MS acknowledges a research grant from the DFG (SCHM2661/3-2). The work of DS is supported by the Tisch Cancer Institute and the PhD Scientist Innovative Research Award. J.M.L. is supported by National Cancer Institute (P30-CA196521), NIDDK (R01 DK128289), US. Department of Defense (CA150272P3), European Commission/ Horizon 2020 Program (HEPACAR, Ref. 667273-2), Accelerator Award (CRUK, AECC, AIRC) (HUNTER, Ref. C9380/A26813), Samuel Waxman Cancer Research Foundation, Centro de Investigación Biomédica en Red (CIBER) – ISCIII, Spanish National Health Institute (SAF2016-76390), Generalitat de
Catalunya/AGAUR (SGR-1358) and the Acadèmia de Ciències Mèdiques i de la Salut de Catalunya i de Balears.

**Conflicts of interest relevant to this work:**

JFD has received consulting fees from Abbvie, Bayer Healthcare, Bristol-Myers Squibb, Falk, Genfit, Genkyotex, Gilead Sciences, HepaRegenix, Intercept, Eli Lilly, Merck, Novartis, Roche. JUM. received honoraria from Roche, Bayer, Ipsen, Merz, AstraZeneca, MSD and Leap-Tx, Eisai. PRG is receiving honoraria from Adaptimmune, Bayer, BMS, AstraZeneca, Sirtex, MSD, Eisai, Ipsen, Roche, Lilly, Guerbet. AV has received consulting fees and honoraria from AstraZeneca, Bayer, BMS, Eisai, Incyte, Ipsen, Janssen, Lilly, Merck, MSD, Novartis, Pierre Fabre, Roche, and Sanofi. TM reports consulting fees from IPSEN, AstraZeneca, Roche, Bayer Healthcare, Adaptimmune, Boston Scientific and Eisai. LRR has received grant funding from Bayer, BTG International, Exact Sciences, Gilead Sciences, GlycoTest, Redhill, TARGET PharmaSolutions and FUJIFILM Medical Systems, and has consulted for AstraZeneca, Bayer, Exact Sciences, Gilead Sciences, GRAIL, QED Therapeutics, and TAVEC. B.M. received consultancy fees from Bayer-Shering Pharma, and speaker fees from Eisai and MSD. MS is receiving honoraria from ERBE, Amgen, Merck and Bayer Healthcare. MWS is receiving consulting fees from Bayer, Eisai and Exelixis. RSF has received consulting fees from AstraZeneca, Bayer Healthcare, Eisai, CStone, Bristol-Myers Squibb, Eli Lilly, Pfizer, Merck, Roche/ Genentech, Exelixis. AV has received consulting fees from Guidepoint, Fujifilm, Boehringer Ingelheim, FirstWord, and MHLife. JML is receiving research support from Bayer HealthCare Pharmaceuticals, Eisai Inc, Bristol-Myers Squibb, Boehringer-Ingelheim and Ipsen, and consulting fees from Eli Lilly, Bayer HealthCare Pharmaceuticals, Bristol-Myers Squibb, Eisai Inc, Celsion Corporation, Exelixis, Merck, Ipsen, Genentech, Roche, Glycotest, Nucleix, Sirtex, Mina Alpha Ltd and AstraZeneca. The remaining co-authors have nothing to disclose related to this manuscript.
Author contributions: Study design: PKH, MP, DS, JML. Patient enrolment/sample and data collection: PKH, FC, MP, PR, JFD, CV, CC, JM, PG, AV, MB, TM, IL, AD, LRR, MAM, BM, DC, VM, FF, JT, BÖ, TM, MS, AB, MWS, MES, RSF. Experiments: PKH, MTM, MP, CAO, MM. Analysis: PKH, MTM, CAO, FC, ST, AV, DS, JML. Drafting of manuscript: PKH, DS, JML. All authors gave intellectual input to the manuscript and have approved its final version.


Word count: 6960
Abstract word count: 260
Number of figures: 6
Number of tables: 1
ABSTRACT

Background and Aims
Single agent anti-PD1 checkpoint inhibitors convey outstanding clinical benefits in a small fraction (~20%) of patients with advanced hepatocellular carcinoma (aHCC) but the molecular mechanisms determining response are unknown. To fill this gap, we herein analyze the molecular and immune traits of aHCC in patients treated with anti-PD1.

Methods: Overall, 111 tumor samples from patients with aHCC were obtained from 13 centers prior to systemic therapies. We performed molecular analysis and immune deconvolution using whole genome expression data (n=83), mutational analysis (n=72) and histological evaluation with an endpoint of objective response.

Results: Among 83 patients with transcriptomic data, 28 were treated in frontline whereas 55 patients were treated after tyrosine-kinase inhibitors (TKI) either in 2nd or 3rd line. Responders treated in frontline showed upregulated Interferon-γ-signaling and MHCII-related antigen presentation. We generated an 11-gene signature (IFNAP), capturing these molecular features, which predicts response and survival in patients treated with anti-PD1 in frontline. The signature was validated in a separate cohort of aHCC and >240 patients with other solid cancer types where it also predicted response and survival. Of note, the same signature was unable to predict response in archival tissue of patients treated with frontline TKIs, highlighting the need for fresh biopsies prior to immunotherapy.

Conclusion: IFN-signaling and MHCII-related genes are key molecular features of HCCs responding to anti-PD1. A novel 11-gene signature predicts response in frontline aHCC - but not in patients pre-treated with TKIs. These results have to be confirmed in prospective studies and highlight the need for biopsies prior immunotherapy to identify biomarkers of response.

Keywords: Hepatocellular carcinoma, biomarkers, predictors of response, Immunotherapy, Anti-PD1, molecular therapies.
INTRODUCTION

Hepatocellular carcinoma (HCC) is a leading cause of cancer related mortality globally and incidence rates are on the rise. At advanced stages, where only systemic therapies are effective, outcomes remain dismal. Until recently, the treatment landscape of HCC was dominated by tyrosine-kinase inhibitors (TKIs), such as sorafenib and lenvatinib that have been able to convey a marginal improvement in survival for the majority of the population.

The use of immune checkpoint inhibitors (ICI) has revolutionized clinical care across cancer types. In HCC, the combination of the anti-PD-L1 agent atezolizumab and the monoclonal antibody (mAB) bevacizumab (anti-VEGF) has elicited an outstanding median overall survival (mOS) of 19.2 months in patients with advanced HCC (IMbrave150 trial) and is now considered standard of care in frontline. Furthermore, new ICI-based combinations are expected to reshape the treatment scenario such as the combination of durvalumab-tremelimumab, which increases overall survival (OS) relative to sorafenib and cabozantinib-atezolizumab, which increases progression-free survival (PFS). These and other combinations currently under investigation are aimed at enhancing the size of the patient subset that derives a benefit from ICI treatment. In this setting, ICIs are commonly regarded as the driving force in improving outcomes whereas drugs like bevacizumab are thought to expand the immune-sensitive population. Indeed, immunotherapy as standalone treatment is able to convey meaningful benefits in patients with advanced HCC: early efficacy results from anti-PD1 inhibitors nivolumab and pembrolizumab demonstrated objective response rates (ORR) between 15-20%. These responses lasted beyond 16 months and are expected to elicit a mOS >26 months, thereby outperforming the new standard of care. However, the comparatively small size of this subset failed to drive a significant advantage for the entire population leading to the failure of phase III trials both in frontline (vs sorafenib) and 2nd line setting (vs. placebo), hence the utilization of combination treatments. The molecular mechanisms that determine response to anti-PD1 in HCC remain elusive. Thus, the development of predictive biomarkers of response to ICI has the potential to address several unmet clinical needs: (I) to enhance survival in patients likely to respond to therapy, (II) to reduce
the risk of treatment-related adverse effects conveyed through combination drugs like bevacizumab and (III) maximize efficacious application and thereby cost-effectiveness of different treatments.

To address these needs, we established an international consortium of referral centers to identify biomarkers of response in patients treated with anti-PD1. We analysed tissue samples from patients subsequently undergoing anti-PD1 treatment for advanced HCC at the histological, mutational and gene expression levels. Patients responding to anti-PD1 in frontline showed higher baseline levels of intratumoral inflammatory signalling. We generated a gene-expression signature capable of capturing responders and validated it in an independent cohort of aHCC patients and four publicly available datasets of solid cancer types comprising >240 patients. Interestingly, the same signature failed to predict response in patients that were pre-treated with TKIs, suggesting that archival tissue may not be appropriate to predict response to immunotherapy in patients previously treated with TKIs as these drugs may modulate response patterns to 2nd line anti-PD1 therapy. Overall, these findings provide a comprehensive picture of the molecular landscape of patients with advanced HCC responding to anti-PD1 and define a novel tool for patient selection in future clinical trials.

MATERIALS AND METHODS

Study population and endpoints

Under the umbrella of an international consortium comprising thirteen centers in the United States and Europe (Table S1), we retrospectively collected samples from 111 patients for this study. Eligible patients were ≥18 years with pathologically confirmed HCC at advanced stage (BCLC stage C) or intermediate stage (stage B) after confirmed progression to locoregional therapies and not amenable to a curative treatment approach. Response assessment was performed at least two months after the initiation of anti-PD1 treatment via modified RECIST (mRECIST\textsuperscript{13}) and at least one untreated lesion was required for inclusion. Patients had compensated liver function and Eastern Cooperative Oncology Group (ECOG) performance score 0-2 as well as otherwise adequate organ and bone marrow function (white blood cell counts ≥2000/μL, platelets ≥50×10\textsuperscript{3}/μL). All patients had archived tissue available obtained from the resection specimen or at the time...
of biopsy prior to systemic therapies and underwent anti-PD1 monotherapy. Patients who had been previously treated with an agent targeting T-cell costimulation or checkpoint pathways (including PD-1/PD-L1) were excluded, as were those receiving anti-PD1 or any other treatment neoadjuvantly prior to resection or in combination with other systemic or percutaneous treatments. Further exclusion criteria were: history of other malignancies, other diseases expected to severely limit life expectancy, brain metastases, history of hepatic encephalopathy or clinically significant ascites that required paracentesis. Patients with fibrolamellar HCC, sarcomatoid HCC, or mixed cholangiocarcinoma-HCC were excluded. The present study was conducted in accordance with the Helsinki Declaration and local laws. The institutional review board (IRB) at each contributing center approved the study protocol. All patients alive at the time of study initiation provided written informed consent enabling to use their archived tissues. Consent for already deceased patients was waived by the local IRBs.

Given that different systemic treatments may alter the tumoral microenvironment to the point that it may impact the efficacy of subsequent therapies\textsuperscript{14}, we stratified patients according to the treatment line in which they received anti-PD1 (Table S2).

The primary endpoint applied for the analysis was best objective response (OR), which was assessed in individual centers using mRECIST criteria defining complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD)\textsuperscript{13}. Response was generally assessed 2-3 months after therapy start and every three months thereafter via either computed tomography (CT-scan) or magnetic resonance imaging (MRI). The secondary endpoints were overall- (OS) and progression-free survival (PFS).

**Immunohistochemistry, transcriptome analysis, CTNNB1 analysis and molecular data availability**

See online supplementary materials and methods.

**Statistical analysis**

Analyses were performed using the R statistical package and SPSS 24.0 (SPPS Inc., Chicago, USA). Correlations between clinicopathological data and molecular features were performed in case of categorical data with Chi\textsuperscript{2} test, whereas continuous data with
non-parametric distribution was assessed by Wilcoxon-rank-sum test. Continuous variables with Gaussian distribution were compared with ANOVA. Survival analysis was performed with Kaplan-Meier estimates and log-rank test with respect to both OS and PFS as well as a Cox logistic regression model. Biomarkers were considered predictive of response or primary resistance to anti-PD1 therapy when two sided p<0.05.

RESULTS

Baseline characteristics and clinical courses

Among the 111 HCC samples collected for the study, 83 cases had enough tissue available for molecular analysis, met all inclusion criteria and were thus included in the transcriptomic analysis (Figure 1A). The time difference between acquisition of the biological sample and initiation of systemic therapies is depicted in Figure S1. Recruited patients were treated with nivolumab (n=67; 80.7%), pembrolizumab (n=14; 16.9%) or tislelizumab (n=2; 2.4%) in either frontline (n=28), 2nd (n=41) or 3rd line (n=14) (Figure 1B). All patient demographics and disease characteristics were well balanced between response types (Table 1). Among the 83 patients, 25 exhibited OR (ORR:30.1%, 3 CR, 22 PR), whereas 21 cases (25.3%) had SD and 37 cases (44.6%) PD as best response. Median time to response was 3.0 months (range 1.7-12.8 months) and responses were very durable with 67% lasting 18 months or longer. Median duration of treatment was 4.9 months, and patients displaying OR had a significantly longer time on therapy than non-responders (18.2 vs. 3.3 months, p<0.001). In terms of outcome, median follow-up was 12.5 months. Responders did not reach mOS during follow up, whereas mOS was 19.5 months and 12.5 months for patients achieving SD and PD, respectively (p<0.001, Figure 1C). Likewise, responders had significantly longer PFS compared to patients with either SD or PD (median PFS [mPFS]: 28.8 vs. 6.2 vs. 2.5 months, p<0.0001) (Figure 1D).

Of the 28 patients in the frontline cohort, 12 exhibited OR (ORR:42.9%). These patients had, expectedly, a significantly better outcome than non-responders both in terms of OS and PFS (p<0.005 and p<0.001, respectively, Figure 2A,B). A detailed description of patients treated with anti-PD1 in 1st line is provided in Table S3.
Molecular features of HCC patients responding to anti-PD1 in front line

Overall, differential expression analysis identified 427 genes significantly upregulated in responders (p<0.01, Table S4), with 140 exhibiting a Fold Change >1.5. Among these, several genes involved in Interferon-γ (IFNγ)-signalling (STAT1, STAT2, IRF1, p<0.0005, p<0.05, p<0.05, respectively, Figure 2C) and antigen-presentation were significantly upregulated in responding patients. This was particularly evident for MHC class II peptides (HLA-DRA, HLA-DQA1, HLA-DMA, p<0.01, p<0.005, p<0.05, Figure 2C,D). Metagenes capturing activation of IFN- and T-cell receptor signalling as well as antigen processing and presentation were, likewise, enriched among responders (FDR<0.001). The same patients showed a significant upregulation in the expression of key cytokines involved in chemotaxis (CXCL9, IL18, p<0.005 and p<0.001, respectively). Gene Ontology (GO) Enrichment Analysis of the top 140 differentially expressed genes confirmed IFNγ signalling, MHC-II assembly and MHC-II dependent antigen presentation as the most overexpressed pathways among responders (Figure 2E, S2A). Gene set enrichment analysis using the Hallmark gene sets confirmed enhanced IFN-signalling in responders (FDR<0.001, Table S5, Figure S2B). Comparison between patients exhibiting disease-control (DCR=OR+SD) and those with PD revealed a significant enrichment in CD274 (PD-L1) expression in DCR patients, although no difference was observed in PD-L1 staining by immunohistochemistry, a discrepancy previously characterized15. Likewise, gene expression of PDCD1LG2 (PD-L2), the alternative ligand to PD-1, was significantly higher in responders (p<0.01), whereas expression of its common receptor PDCD1 (PD1) was markedly increased among patients with PD (p<0.05) (Figure 2C).

We next sought to correlate clinical response to anti-PD1 therapy with previously established molecular classes of HCC (Figure 3A) including the recently characterized HCC Inflamed class16, which further refines our previously published Immune class of HCC17. The inflamed class entails three subtypes, named Active, Exhausted, Immune-like that all share a microenvironment with increased interferon-signalling. Interestingly, patients belonging to the HCC Inflamed class showed a higher rate of OR compared to the other classes Intermediate and Excluded (7/9, vs 5/19, p=0.01), whereas differences
in PFS showed a non-significant trend (p=0.068, Figure S2C). Patients with an aggressive and proliferative HCC phenotype (classes S1 and S2) had markedly longer PFS when treated with anti-PD1 compared to the rest (p=0.017, Figure S2D). Next, we evaluated previously reported signatures and biomarkers of response to anti-PD1 therapy in our dataset (Table S6). Interestingly, we observed that several signatures such as the IFN signature, POPLAR and the Inflammatory signature were significantly enriched in HCC responding patients (p<0.01, p<0.01, p<0.05, respectively, Figure 3B). In terms of outcome, both IFN- and the Inflammatory signature were associated with longer PFS (p=0.023 for both, Figure 3C,D) whereas no differences were observed for POPLAR or the cytolytic activity signature (Figure 3E,F). However, none of the signatures was able to predict significantly longer OS (Figure 3G-J). A summary of the performance of these signatures is provided in Table S7. When considering histological markers such as the richness of the immune infiltrate, Tertiary lymphoid structures signature and PD-L1 expression as well as tumor mutational burden inferred through a gene signature no positive correlation with response to ICIs was observed (Figure S3A,C).

A novel 11-genes signature accurately predicts response to anti-PD1

Given the paucity of studies identifying candidate biomarkers for anti-PD1 in HCC and that none of the previously reported signatures was able to predict both PFS and OS, we then developed a gene expression signature capable of discriminating responding from non-responding patients (see supplementary methods). The resulting 11-gene set, hereafter named IFNAP-signature (Interferon and antigen-presentation, Table S8), comprises genes involved in IFN-γ signalling (STAT1, GBP1), antigen presentation (B2M, HLA-DRB5, HLA-DRA) and chemotaxis (CXCL9, Figure 4A). Most of these genes were not shared with other published immune response signatures (Figure S4B) underscoring the unique composition of IFNAP. The IFN signature and IFNAP shared three individual genes, all of which were predictive of OR and PFS. Likewise, the non-overlapping genes in IFNAP were linked with OR and PFS, whereas the remaining genes in the IFN signature where not (Figure S5A-C). Patients with high expression of IFNAP (n=9, defined as those within the upper tertile, see supplementary methods) had superior outcomes both with
regard to PFS (p=0.035) and OS (p=0.039, Figure 4B,C). ROC analysis indeed revealed IFNAP as the most efficient geneset at discriminating responding from non-responding patients with an AUC of 0.87 (Figure 4D).

We next sought to investigate the robustness of the IFNAP signature by testing its stability across different regions within a given tumor to investigate whether intratumoral heterogeneity, which may cause differences in regional adaptive immune responses, may compromise the reproducibility of signature expression. For this purpose we re-analyzed a cohort published by our group including 30 HCC samples from 15 patients with tumors >4cm23, we found expression between two distinct regions of the same tumor to be very stable and 90% of cases had the same expression category (low/high) in both samples. Indeed, correlation of IFNAP between two regions of a given tumor was significant (R=0.77, p<0.001, Figure S4C).

We tested IFNAP in four independent datasets (see supplementary methods) comprising 240 patients with either NSCLC24,25, HNSCC24 or melanoma24,26,27 treated with anti-PD1/anti-PD-L1. In the first dataset24, patients with OR had significantly more often high IFNAP expression, which was, moreover, associated with longer mPFS (55% vs. 24.4%, p=0.017 and 6.9 vs. 2.8 months, p=0.039, respectively, Figure 4E-H). Likewise, in the second dataset25 high IFNAP expression was associated with response and longer mPFS (75% vs. 15.8%, p=0.006 and 8.6 vs. 1.2 months, p=0.006, respectively, Figure 4G-H). In the third dataset27, OR was again associated with high IFNAP expression, (42.5% vs. 27.4%, p=0.043, Figure S6A), which in turn predicted longer mPFS and mOS (13.3 vs. 3.2 months, p=0.011 and NR vs. 19.7 months, p=0.033, respectively, Figure S6B,C). Finally, IFNAP also predicted higher response (46.7% vs. 15.6%, p=0.039, Figure S6M) in the fourth dataset26. Of note, none of the previously published signatures consistently predicted response or PFS in any of the datasets (Figure S6D-L,N-P and Figure S7A-F, I-L) with the exception of the IFN signature in the Jung et al dataset (Figure S7G-H). In summary, the IFNAP signature was able to capture responders to anti-PD1 pre-treatment across cancer types and was associated with longer PFS and OS whereas none of the previously published signatures was capable of consistently eliciting the same significant differences. This is of note as several of these signatures were designed in tumors that
are investigated in the validation datasets\textsuperscript{18,19}. In these, patients were treated with anti-PD1 both in frontline as well as in second line. However, unlike in our cohort, none of the patients underwent TKI treatment prior to immunotherapy and most tissue samples were obtained directly prior to the initiation of anti-PD1 therapy.

Finally, we tested the ability of IFNAP to predict response and longer survival in a dataset of patients treated with either single-agent ICI (n=13, nivolumab) or combination treatment (nivolumab/ipilimumab or spartalizumab/sabatolimab, n=11)\textsuperscript{28}. High expression of IFNAP assessed by nanostring was associated with significantly longer OS and a trend towards higher OR (Figure S8A-B) to nivolumab but not to combination treatment (Figure S8C-D) suggesting molecularly distinct mechanisms of response for the combination.

The IFNAP gene signature captures a unique immune microenvironment

Since the increased expression of IFN- and AP-related genes is not unequivocally associated with a specific cell type but can be conferred through both tumoral and immune cells, we next characterized the immune infiltration in patients with high IFNAP expression. Strikingly, patients with high and low IFNAP expression were not different in terms of actual immune cell infiltration quantified on H&E stained slides (see methods) both in the intratumoral area and at the invasive margin (Figure 5A). We next hypothesized that the microenvironmental composition rather than overall infiltration may drive response to anti-PD1 in HCC. Thus, we performed virtual microdissection using CIBERSORT\textsuperscript{x} and found a significant upregulation of plasma cells, CD4-memory activated Tcells and M1 macrophages in patients with high expression of IFNAP (Figures 5B, S9). Conversely, patients with low IFNAP showed a significant increase in the infiltration of immunosuppressive regulatory Tcells (Tregs, p=0.001). Indeed, expression of IFNAP showed a negative correlation with expression of Tregs and of Forkhead-Box-Protein P3 (FOXP3, Figure 5C), a transcription factor active in Tregs which has been previously implicated in driving immunosuppression across cancer types and is linked with hyperprogression after anti-PD1\textsuperscript{30}. In our dataset, low expression of Tregs or IFNAP, defined by the 1\textsuperscript{st} tertile, was associated with markedly lower PFS (4.9 vs. NR and 3.6 vs. 28.8 months, p=0.012 and p<0.001, respectively, data not shown). We also considered
other features associated with primary resistance to anti-PD1 and found significant negative correlations between IFNAP and PDCD1 expression (Figure 5C). Interestingly, all these markers of immunosuppression were highly correlated with each other and were negatively correlated to all genes of the IFNAP signature (Pearson correlation, Figure 5D). Overall, these data indicate that immunosuppressive expression programs predict poor outcome after anti-PD1 therapy. The presence of Tregs in the microenvironment may be one of the key factors eventually driving resistance while the other factors could represent downstream effects of this microenvironmental composition.

**CTNNB1 mutational status is not a dominant feature to predict resistance to anti-PD1 therapy**

Mutations in the WNT-CTNNB1 pathway have been implicated in driving resistance in a murine model of HCC. We then investigated whether the presence of CTNNB1 mutations was able to predict primary resistance to anti-PD1. To this end, we correlated treatment response with tumoral mutational status in 23 cases of frontline-treated patients. We found 4 of 11 responders (36%) and 6 out of 12 non-responders (50%) to have mutations in exon 3 of CTNNB1, the dominant hotspot, thereby showing no significant differences in response rates (Figure 5E). We considered that patients with CTNNB1 exhibited less durable responses than non-mutated patients but no differences were observed in PFS and OS. Likewise, no difference in PFS and OS was seen among non-responding patients based on mutational status (Figure S10). Next, we compared the gene expression profile of CTNNB1 mutated patients that exhibited response (n=4) to those patients with mutations that did not (n=6). We observed a trend towards increased inflammatory signalling as captured by the cytolytic activity gene signature in those patients with mutations that responded. Moreover, the same patient subset demonstrated an upregulation in genes associated with an active immune response (GZMA, CXCL9; one-sided p<0.05).

In summary, CTNNB1 mutational status did not predict resistance to therapy. A trend towards more inflammatory signalling in responders despite the presence of mutations hints at a more intricate role of CTNNB1 in this scenario. While previous studies have
shown discrepancies in terms of the role of \textit{CTNNB1} as a driver of immune exclusion\textsuperscript{31,33,34}, our findings provide an explanation to reconcile these inconsistencies. Indeed, our data suggest that patients with \textit{CTNNB1}-driven immune exclusion may be prone to resistance. However, in tumors where this profile is overcome by unknown mechanisms to establish an inflamed microenvironment, the conducive effects of IFN-signaling and the intact antigen-presenting machinery may outweigh the impact of \textit{CTNNB1} mutations.

\textbf{Prior treatment with TKIs may influence response to subsequent anti-PD1 in 2\textsuperscript{nd} line}

Overall, 55 patients underwent anti-PD1 treatment as 2\textsuperscript{nd} (41 cases) or 3\textsuperscript{rd} line (14 cases) therapy after previous exposure to TKIs (54 sorafenib, 1 lenvatinib, Table S9). In all but two of these cases, though, histology was obtained prior to first line therapy. Overall, the ORR was 23.6\%, and as in frontline treated patients, responders had both markedly longer OS and PFS (p=0.047 and p<0.0001, respectively, Figure 6A-B). In this setting, neither IFNAP (n=18 patients in 2\textsuperscript{nd}/3\textsuperscript{rd} line) nor other previously reported signatures were significantly enriched among patients with OR (Figure 6C, S11A,D,G,J). This translated to clinical outcome, where no differences were observed between patients with high and low expression of these signatures (Figure S11B,C,E,F,H,I,K,L). Likewise, histological severity of the immune infiltrate and inferred presence of Tertiary lymphoid structures signature\textsuperscript{21} and high tumor mutational burden (TMB) were not linked to response in patients treated with anti-PD1 in 2\textsuperscript{nd} line either (Figure S3B,C).

We thus considered that TKIs may impact the success of subsequent anti-PD1 therapy in a way that renders some tumors that would be expected to respond to anti-PD1 in frontline no longer responsive after prior TKI therapy. Conversely, a subset of tumors that would be expected to exhibit resistance to anti-PD1 when treated in frontline did respond when pretreated with TKIs. In an exploratory analysis, we investigated factors that may guide whether or not TKI therapy is conducive for subsequent anti-PD1 treatment. Patients with low inflammatory signaling and resistance to therapy (IFNAP low NR) showed an upregulation in metabolic signaling pathways compared with patients with low inflammatory signaling that did respond (IFNAP low OR, Figure S12B) and retained the
significant enrichment in Tregs infiltration by CIBERSORTx (Figure S12C). Conversely, patients with low IFNAP expression and response showed a marked increase in CD4 naïve T cell infiltration. Overall, this data suggested that severe infiltration of regulatory T cells may impede anti-PD1-mediated anti-tumoral immunity even after TKI therapy, as this feature was maintained both in frontline and 2\textsuperscript{nd}/3\textsuperscript{rd} line treated patients. Indeed, markedly worse PFS was observed in the top 20% of patients that harboured the highest infiltration in Tregs in both frontline and 2\textsuperscript{nd}/3\textsuperscript{rd} line (Figure 6C, D-E). This same subset of patients presented a significant enrichment in the expression of SOCS1 and SOCS3, key antagonists of JAK/STAT signaling and thus inhibitors of the intracellular IFN-response pathway (Figure 6C). In keeping with this, the same subset featured significant downregulation in key genes involved in IFN-signaling and an active antigen presenting machinery.

In the absence of human datasets featuring serial biopsies to investigate the distinct effect of TKIs on the tumoral microenvironment, we explored a recently published murine model in which HCC-bearing mice were treated with either lenvatinib or placebo (Figure S12D, see supplementary methods). Comparative gene expression analysis of mice treated with Lenvatinib for two weeks revealed a significant enrichment in inflammatory signaling by TKIs as captured by higher expression of IFNAP and the IFN signatures (Figure S12E). Cellular subsets, such as CD4 effector memory cells, that we linked to response to anti-PD1, were, likewise, upregulated after Lenvatinib treatment. Overall this data suggests that TKIs may modulate response to anti-PD1 by altering microenvironmental signalling.

Overall, our findings suggest that fresh tissue should be obtained directly prior to the initiation of a given treatment to enable precision oncology as prior lines of systemic therapy compromise the readout quality of biomarkers. Our data indicates a patient subset, characterized through severe Treg infiltration and overexpression of immune-evasion related genes that is linked to poor outcomes when treated with anti-PD1 both in frontline as well as in 2\textsuperscript{nd}/3\textsuperscript{rd} line.

**DISCUSSION**
The present study represents a comprehensive characterization of the molecular patterns associated with response and resistance in patients with advanced HCC treated with anti-PD1. Herein, we identified IFN-signalling and AP-related genes to be associated with OR whereas presence of Tregs and pathways associated with immunosuppression are linked to resistance. We developed an 11-gene expression signature capable of predicting response to anti-PD1 in HCC and other solid cancer types when treated with anti-PD1 in the frontline setting. When testing the signature in samples from patients pre-treated with TKIs, we found that neither our signature nor previously reported inflammatory markers predict outcomes to 2nd or 3rd line anti-PD1 therapy suggesting that prior lines of therapy may impact the efficacy of subsequent anti-PD1.

In recent years, several predictive biomarkers of response and resistance to systemic therapies have entered the clinical space. Regarding anti-PD1 therapy, the only FDA-approved biomarkers are high TMB and microsatellite-instability across cancer types. The benefit conveyed by these biomarkers is limited in magnitude (<3% of HCCs) underscoring the need for more refined testing. Earlier studies in melanoma and lung cancer have observed an increase in T-cell infiltration in the tumor microenvironment and enrichment of IFNγ-signalling in patients responding to anti-PD1 therapy\(^\text{18, 19}\). While this observation has been consistently confirmed in early on-treatment samples collected 2-4 weeks after therapy start\(^\text{35}\), results in samples collected before the initiation of therapy are conflicting\(^\text{26}\).

Among the most relevant findings of our study, we identified a gene expression signature – IFNAP – that predicts response and survival to frontline anti-PD1 in aHCC. Notably, it outperformed previously published signatures of response and was the only one to predict significant increases in ORR, OS and PFS in our dataset as well as in an aHCC validation cohort and four expression datasets from other solid cancer types across different platforms (Nanostring, microarray, RNA-seq). IFNAP identified responders independent of the etiology of the underlying liver disease, where 4/5 responders without viral hepatitis had high expression of the signature. This is particularly relevant in light of a recently published report that draws the efficacy of anti-PD1 therapy in patients with NASH into question\(^\text{36}\). The composition of IFNAP reflects key biological pathways involved in T-cell
directed therapies: (i) Interferon-signalling and (ii) antigen-presentation, which are readouts of nascent cancer cell immunogenicity, that can be leveraged through immunotherapy. IFNAP includes genes such as B2M, whose loss of heterozygosity has been implicated as a mechanism of resistance to anti-PD1, and CXCL9 as well HLA-DRA that have been linked to response in melanoma. In our dataset, analysis of the immune infiltrate suggested that the composition rather than overall infiltrate might drive response to immunotherapy. Specifically, CD4+ naïve T cells were consistently upregulated in patients with high IFNAP expression, whereas Treg infiltration was negatively correlated with IFNAP. The presence of immunosuppressive Tregs and their active transcription factor FOXP3 may in this regard be an impediment towards initiating antitumoral immunity. A recent biomarker analysis of HCC patients treated with atezolizumab and bevacizumab in clinical trials identified that increased IFNy-signalling, active antigen-presentation and low Treg/Effector-T-cell ratio were linked to response. In addition, patients with high Treg infiltration experienced a significantly stronger benefit from combination compared to atezolizumab monotherapy, suggesting synchronous application of ICI with anti-angiogenics may help in overcoming severe Treg infiltration as a driver of resistance to ICI monotherapy.

Several investigations in melanoma have shown genetic alterations in the WNT-CTNNB1 pathway to be a tumor-intrinsic driver of immune exclusion and resistance to anti-PD1. In HCC, a preclinical study suggested that CTNNB1 mutations conveyed defective recruitment of dendritic cells and subsequently impaired cytotoxic T-cell function. These effects were reverted upon overexpression of CCL5. One cohort study supported this correlation in patients that underwent biopsy prior to treatment, whereas another did not identify CTNNB1 mutations in liquid biopsy impacting PFS. Our results point towards the fact that CTNNB1 mutations alone are not associated with resistance, although the underlying biological mechanisms remain elusive. Those patients with inflammatory signalling counterbalancing CTNNB1–related immunosuppression showed a trend to better OR, as opposed to those where CTNNB1 mutations was the dominant molecular feature determining lack of response to anti-PD1. In the former cases, other signalling pathways such as IFN-signalling and an active antigen-presenting machinery may overcome CTNNB1-mediated immune exclusion and thus facilitate response.
Finally, the aforementioned differences in expression profiles between responding and non-responding patients were no longer evident in those patients receiving TKIs between sample acquisition and immunotherapy start. This finding could be due to the longer time elapsed between tissue acquisition and anti-PD1 treatment in the 2nd/3rd line when compared to the frontline which may increase the odds of molecular events contributing to immunosuppression, although this is unlikely given the relative stability of driver events during cancer evolution\textsuperscript{40}. An alternative hypothesis would be that treatment with TKIs may influence how a patient responds to anti-PD1 therapy in subsequent treatment lines. In the absence of serial biopsies, the molecular mechanisms that guide the impact of TKIs on other treatments remain unknown and it is unclear as to whether an inflamed microenvironment pre-sorafenib remains inflamed thereafter or if the effect of the TKI may ameliorate inflammatory signalling in these tumors.

Data from murine model suggests\textsuperscript{41, 42} that TKIs may overall increase inflammatory signalling within the tumor and induce a shift in the composition of the microenvironment. However, it needs to be acknowledged that these models are not fully reflective of human disease course since the molecular analyses were performed on animals during TKI treatment while humans are generally not exposed to anti-PD1 before resistance to TKIs. As previous studies have shown that while sorafenib sensitive tumors display an increase in inflammatory signaling and an enhanced antigen-presentation apparatus, resistance in turn is associated with a less rich T cell infiltration and less overall inflammatory signaling within the tumor\textsuperscript{42}. Likewise, a recent biomarker companion study for a phase I clinical trial aiming at converting locally advanced disease to resectable HCC through neoadjuvant cabozantinib and nivolumab confirmed heterogeneous expression of TKI targets and inflammatory markers based on response status\textsuperscript{43}.

Our data implies that responders in different treatment lines are different populations with some overlap. Conversely, we have identified a subset of patients that exhibit resistance to anti-PD1 regardless of whether treatment is administered in frontline or after exposure to TKIs. This subset was characterized through an increase in Treg infiltration and expression of genes that are direct inhibitors of active JAK/STAT signalling. Overall, our data opens up the enticing perspective that more HCC patients could respond to anti-
PD1 therapy through selective pre-treatment/or combination with TKIs, although some patients may not be suitable for anti-PD1 in any case. Our data ultimately calls for the need of biopsies prior to anti-PD1 treatment start to enable biomarker-based precision oncology regardless of treatment line.

Several limitations of this study need to be addressed: first, the distinction between therapy lines diminishes the sample size considerably and limits the power of the study despite recruitment from 13 international referral centers. The observed response rate of 43% in the first-line cohort is certainly above the expected 15-20% response rate and is a reflection of the inclusion criteria of a minimal duration of 2 months of treatment to evaluate response. While this naturally increases the proportion of responders, it also increases the power of our biomarker analysis. In addition, the use of mRECIST response criteria likely contributes a small further increase in the ORR. Second, the lack of serial biopsies between systemic treatments precludes a refined analysis on how precisely TKI therapy alters the microenvironment and impacts efficacy of subsequent immunotherapy. Finally, validation of IFNAP could only be performed in a comparatively small HCC dataset as well as cohorts with other cancer types. Therefore, validation of IFNAP in future larger HCC cohorts remains a critical unmet need particularly in light of the limited number of cases that were used for the construction of the signature.

In summary, our study defines the key molecular drivers of response to anti-PD1 in HCC. We generated and validated a signature recapitulating these pathways that predict response and longer survival in HCC and other cancer types and therefore has potential to maximize the efficiency of anti-PD1 application. The final value of this signature needs to be explored within phase III investigations. In patients treated with 2nd and 3rd line anti-PD1, prior TKI therapy likely impairs the predictive potential of the IFNAP signature, although further studies will be required to clarify the reasons for this observation.

REFERENCES


## Table 1

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<th>Progressive disease (n = 37)</th>
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<tr>
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<td>63 (22-79)</td>
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<td>Gender, male (%)</td>
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<td>17 (81.0)</td>
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<td><strong>Etiology (%)</strong></td>
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<td>HBV</td>
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<td>A</td>
<td>72 (88.9)</td>
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<td>B</td>
<td>9 (11.1)</td>
<td>1 (4.8)</td>
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<td>Resection</td>
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<td><strong>Median time to response in months (range)</strong> ##</td>
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<td>3 (1.7-12.8)</td>
<td>3 (1.7-12.8)</td>
</tr>
</tbody>
</table>

*BCLC – Barcelona clinic liver cancer; AFP – Alpha fetoprotein; *Data missing from 2 cases; **Data missing from 31 cases; ***Data missing from 3 cases; #Data missing from one case; ##Data missing from 7 cases*
Figure 1. Cohort overview and outcomes. (A) Study flowchart: Of the 111 samples collected for this study, 83 cases, treated with anti-PD1 in either frontline or after exposure to TKIs were eventually included in the transcriptomic analysis. (B) Alluvial plot showing response patterns based on treatment line. The numbers in the boxes represent the number of patients with that specific response (C,D) Kaplan-Meier (KM) estimates of all patients included in the transcriptomic analysis are shown for OS (C) and PFS (D) based on whether patients exhibited objective response (OR), stable disease (SD) or progressive disease (PD). P values in KM curves represent log-rank tests.

Figure 2. Upregulation of inflammation and antigen-presentation associated genes in responders. (A,B) KM estimates for OS (A) and (PFS) for all 28 patients treated with anti-PD1 in frontline based on whether patients exhibited OR or non-response (NR). (C) Heatmap of gene expression analysis based on observed response types. Each signature or individual gene is significantly upregulated in one response subgroup relative to the others, whereas no differences were observed regarding PD-L1 by IHC. (D) Volcano plot showing differentially expressed genes in responders compared to non-responders. Genes differentially expressed at p<0.05 are depicted in red, all others in orange. (E) GeneOntology gene set enrichment analysis of differentially expressed genes using the biological processes classification. P values in KM curves represent log-rank tests.

Figure 3. Association of previously reported gene signatures and HCC molecular classes with response and resistance to anti-PD1. (A) Circular classification plot integrating response types with molecular classes of HCC. Each sector represents one patient. Significant enrichment of the S1/2 classes and the Inflamed HCC subgroup is observed in responders. (B) Boxplot comparison for the expression of previously reported gene signatures based on observed response. (C-J) KM estimates for PFS (C-F) and OS (G-J) based on expression of previously reported signatures. P values in boxplot comparison represents Mann-Whitney test, while those in the KM curves represent log-rank tests.

Figure 4. Generation and validation of expression signature associated with response. (a) Heatmap of genes incorporated in the IFNAP signature. (B,C) KM
estimates for PFS (B) and OS (C) are shown based on expression of IFNAP. (D) Receiver operating characteristic (ROC) curve is shown for IFNAP and previously characterized signatures of response. (E-F) Validation of IFNAP in two independent datasets of anti-PD1/anti-PDL1 treated patients with melanoma, non-small cell lung cancer (NSCLC) and head and neck squamous cell cancer (E,G) and NSCLC (F,H), respectively. Patients with response showed marked enrichment in IFNAP (E,F) which was associated with longer PFS (G,H). P values for KM analysis derive from log-rank test whereas those in the barplots represent 2-sided Chi² test.

**Figure 5. Characterization of IFNAP and correlates of resistance to anti-PD1.** (A) Histological assessment of the immune infiltrate, applying a previously characterized semi-quantitative score, in the intratumoral compartment and at the invasive margin. (B) Boxplot representation of virtual-microdissection with CIBERSORTx based on IFNAP expression. (C) Correlation of IFNAP expression with previously characterized resistance markers. (D) Correlation heatmap with unsupervised clustering of factors associated with response and resistance to anti-PD1 therapy. (E) Heatmap of patients treated in frontline with anti-PD1 ordered by response and CTNNB1 mutational status. No differences in response rates were observed, while a trend towards increased inflammatory signaling in responders with CTNNB1 mutations compared to non-responders with mutations was noted. P values in boxplot comparison represents Mann-Whitney test, while those in the correlation plots represent Pearson tests.

**Figure 6. TKI therapy compromises predictive potential of response-signatures.** (A-B) Kaplan-Meier estimates for PFS and OS in patients treated with anti-PD1 in 2nd/3rd line. (C) Heatmap of patients treated with anti-PD1 in 2nd and 3rd line highlights inability of previously characterized markers to capture responders to anti-PD1 after TKI therapy. (D-E) Forest plots showing log Hazard ratios from a Cox regression model for PFS defines high infiltration of Tregs (Top 20%) as a poor prognostic marker in patients treated with anti-PD1 both in frontline (D) and after exposure to TKIs (E). P values in KM curves represent a log-rank test.
Figure 1

A
Genomic analysis

111 Cases recruited from 13 centers
- 40 treated in Frontline
- 54 treated in 2nd line
- 17 treated in 3rd line

Reasons for case removal
- Insufficient tissue available (n=23)
- Failed QC (n=4)
- Response assessment after 2 weeks (n=1)

83 Cases for transcriptomic analysis

Frontline (n=28)
- CTNNB1 mut.
- Analysis (n=23)
- IHC (n=16)

2nd/3rd line (n=55)
- CTNNB1 mut.
- Analysis (n=44)
- IHC (n=40)

B

C
OS after anti-PD1

Proportion of patients

Time in months

Number at risk

D
PFS after anti-PD1

Proportion of patients

Time in months

Number at risk

Response
OR
SD
PD

28
Figure 2

A

OS in frontline

Proportion of patients

0.00 0.25 0.50 0.75 1.00

0 10 20 30 40 50

Time in months

P = .0018

Number at risk

16 8 2 2 1 1

12 12 6 4 3 3

0 10 20 30 40 50

Time in months

B

PFS in frontline

Proportion of patients

0.00 0.25 0.50 0.75 1.00

0 10 20 30 40

Time in months

Response

NR OR

Number at risk

16 1 0 0 0 0

12 10 5 2 1 0

0 10 20 30 40

Time in months

C

Response

Response Type

Etiology

MacrorvascularInv.

ExtrahepaticDis.

AFP

Reactome_TCR_signalling

Reactome_IFN_

Signal

KEGG_Antigen_Troc_Present

Biocarta_MHC_pathway

D

E

Antigen proc. and present. of peptide

Antigen proc. and present. of peptide via MHC class II

IFN-gamma-med. signaling pathway

Cellular response to interferon-gamma

T cell receptor signaling pathway

MHC class II protein complex assembly

- log10 (FDR)
Figure 4

A

B

C

D

E

F

G

H
Figure 5

A

Infiltrate Tumor

Number of patients

0 1 2 3 4

Infiltrate Border

Number of patients

0 1 2 3 4

IFNAP

Low  High

B

Plasma cells

CD4 mem. act. Tcells

0.15

0.10

0.05

0.00

Low  High

P = .021

P = .004

M1 macrophages

Monocytes

0.35

0.30

0.25

0.20

0.15

0.10

0.05

0.00

Low  High

P = .00041

P = .048

Tregs

IFNAP

Low  High

P = .00032

C

R = -0.86, P = 3.8e-06

R = -0.59, P = .001

FoxP3 expression

IFNAP expression

D

D

Response

OR

SD

PD

E

Response, Type

CR

PR

SD

PD

CTNNB1.status

Mutated

WT

Category

WNT signalling

Response signatures

Immune-related

Response.

Type