Harnessing the immune-mediated cancer field as chemopreventive strategy for hepatocellular carcinoma

Short-title: Immune-mediated field cancerization as target for HCC prevention

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Abbreviations: ALT: alanine aminotransferase; α-SMA: α-smooth muscle actin; AKT: protein kinase B; AST: aspartate aminotransferase; DEN: diethylnitrosamine; CCl4: carbon tetrachloride; EMT: epithelial-mesenchymal transition, ERK: extracellular signal-regulated kinase; FC: Fold change, FDR: false discovery rate; FFPE: formalin-fixed paraffin-embedded; FGFR: fibroblast growth factor receptor; GSEA: gene set enrichment analysis; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; HSC: hepatic stellate cell; ICF: immune-mediated cancer field; IPA: ingenuity pathway analysis; mo: months; NTP: nearest template prediction; PDGFR: platelet-derived growth factor receptor; ssGSEA: single sample gene set enrichment analysis; TKIs: tyrosine kinase inhibitors; Treg: regulatory T cells; qRT-PCR: quantitative real-time polymerase chain reaction; VEGFR: vascular endothelial growth factor receptor.

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ABSTRACT

Background & aims: Cirrhosis and chronic inflammation precede hepatocellular carcinoma (HCC) development in ~80% of cases. We sought to understand the molecular and immune-related features governing the chronic inflammation from which HCC arises, as a prelude to optimizing chemoprevention strategies.

Methods: Gene expression profiling of non-tumor liver tissues from 392 early HCC patients [training (n=167); validation (n=225)] was analyzed to characterize the immune features of the surrounding field. A 172-gene signature capturing the immune-mediated cancer field (ICF) was then tested in a cohort of cirrhotic patients (n=216; median follow-up 10 years) to predict risk of HCC development and outcome. The preventive efficacy of targeting this ICF was assessed in a chemically-induced murine model of chronic liver damage and hepatocarcinogenesis.

Results: An immune-mediated cancer field of the non-tumoral liver was identified in 60% of early HCC cases and in 50% of cirrhotic patients without HCC. This immune field effect comprised three distinct subtypes: 1) *High Infiltrate-ICF* with increased effector T cells; 2) *Immunosuppressive-ICF* with stromal activation and TGF-β signaling, and; 3) *Pro-inflammatory-ICF* with up-regulation of IFN-γ signaling. The *Immunosuppressive*-ICF (10% of cirrhotic patients)(HR:2.41; 95% 1.21-4.80) – as well as platelet count <100,000/mm^3– were independent predictors of HCC development in cirrhotic patients. Nintedanib, an anti-inflammatory and anti-angiogenic kinase inhibitor, reverted this pro-carcinogenic field and significantly delayed HCC onset in preclinical models.

Conclusions: An immunosuppressive *milieu* in non-tumor liver heightens the risk of HCC development. Targeted therapies can revert this pro-carcinogenic field and prevent HCC development in preclinical models. These data establish a rationale for exploring chemopreventive strategies in patients at-risk of HCC development.

Keywords:

Cancer field, immunosuppression, hepatocellular carcinoma, chemoprevention

INTRODUCTION

Liver cancer is the fourth leading cause of cancer-related mortality worldwide[1]. Hepatocellular carcinoma (HCC) accounts for more than 90% of liver cancers and is the main cause of death in patients with cirrhosis[2,3]. HCC cases arise from chronic liver inflammation, fibrosis and eventually cirrhosis in 70-80% of cases[2]. In developed countries, curative treatments are feasible in 30-40% of cases, but recurrence is high and no effective adjuvant therapies are available[2,4]. In addition, ~40-50% of patients are diagnosed at advanced stages when currently approved molecular therapies yield limited survival benefits (~1 year)[5–8]. Despite recent advances in the management and clearance of HCV infection, there is an unmet need for early detection and application of chemopreventive approaches in patients at high-risk of HCC development.

To date, there are no established preventive strategies for HCC in patients at risk beyond prevention with anti-viral therapies[9,10]. Once cirrhosis is established, anti-viral therapies reduce but do not eliminate the risk of HCC[4,11,12]. Individual risk assessment is a key first step in the successful development of any chemopreventive strategy. In this regard, increasing evidence suggests the existence of the so-called “cancer field-effect” or field cancerization which consists of predisposing oncogenic and inflammatory signals occurring during chronic liver injury and ultimately leading to malignant transformation[13–15]. Gene signatures derived from the cirrhotic tissue adjacent to HCC tumors have been designed to predict poor outcome, particularly in HCV-infected cirrhotic patients at higher risk of HCC development[13,16–19]. Overall, these studies support the feasibility of using molecular scores of the carcinogenic field to identify patients at high risk of HCC development. However, the pro-carcinogenic roles of inflammation and immune response in the context of the field cancerization have been poorly explored. Understanding the immune features governing the unresolved cancer field-effect is crucial for identifying potential therapeutic targets in patients at high risk of HCC development.

In this study, the analysis of the inflammatory *milieu* that characterizes the underlying liver disease in which HCC tumors arise has led to the identification of an immune-mediated cancer field (ICF) in 60% of early HCC patients and 50% of cirrhotic patients without HCC. This ICF comprises three distinct molecular subtypes including the *High Infiltrate-ICF* subtype with increased infiltration of effector T cells, the *Immunosuppressive-ICF* subtype with activation of stroma and TGF-β signaling, and the *Pro-inflammatory-ICF* subtype with up-regulation of IFN-γ signaling. These immune profiles, particularly the presence of the *Immunosuppressive-ICF* cancer field, predicts increased risk of HCC development in cirrhotic patients. Inhibition of this pro-

carcinogenic field with nintedanib, an anti-inflammatory and anti-angiogenic kinase inhibitor approved in pulmonary fibrosis, significantly delays HCC onset in a mouse model of chronic liver damage and hepatocarcinogenesis. Overall, our study provides the rationale to explore chemopreventive strategies in cirrhotic patients at high-risk of HCC development.

MATERIALS AND METHODS

Human cohort

For the purpose of the study, gene expression data from a cohort of 167 surgically resected fresh-frozen samples (Heptromic dataset, GSE63898) with matched tumor and adjacent non-tumor tissue were analyzed. All samples were previously collected (1998-2008) in the setting of the HCC Genomic Consortium upon institutional review board approval. Full description of the cohort and RNA profiling data are available in previous publications[20,21]. **Supplementary Table 1** provides a summary of the clinical-pathological variables of the samples used in the current study (referred as training cohort, n=167). Validation of the identified molecular profiles was then performed in an independent set of 225 adjacent non-tumor liver tissues previously characterized by our group (GSE10143)[13]. Finally, for the purpose of identifying those non-neoplastic patients at higher risk of HCC development and most likely to benefit from chemopreventive strategies, our findings were evaluated in a previously characterized cohort of patients with early cirrhosis (n=216, GSE15654)[16] and a publicly available dataset of fibrotic liver tissues (n=124, GSE84044)[22].

Modeling the immune-mediated cancer field

Enrichment scores of 4872 gene sets that represent cell states and perturbations of the immune system (Collection C7 of MSigDB, Broad Institute)[23] were calculated by Single-sample Gene Set Enrichment Analysis (ssGSEA) in the non-tumor liver tissue of the study cohort. Next, unsupervised clustering analysis by non-negative matrix factorization (NMF consensus)[24] method was performed to identify the presence of an immune-mediated cancer field. To characterize the molecular features of those samples presenting an ICF and to identify different immune-mediated field subtypes, a second unsupervised clustering was performed using ssGSEA scores obtained for a curated set of gene signatures representative of individual cell types[25,26] (lymphocytes, macrophages, dendritic cells, mast cells, neutrophils, and eosinophils), cancer immune-related signaling pathways[27] (infiltrating lymphocyte, TGF-β response, IFN-γ

response, macrophage activation, and wound healing), and inflammation- or immune-specific biological processes (Hallmark collection of MSigDB, Broad Institute).

Generation of an immune-mediated field gene signature

An ICF field gene signature was generated using top differentially expressed genes in each molecular group (FDR<0.05; Fold-change ≥2). The ability of the gene signature to capture the key molecular features of the immune-mediated cancer field subtypes was then validated in an independent dataset using Nearest Template Prediction (NTP) analysis using p-value<0.05 for defining significant prediction (Gene Pattern modules)[28].

Molecular characterization of the ICF subtypes and identification of candidate therapies

Enrichment of additional molecular pathways and gene expression signatures in the ICF subtypes was evaluated using GSEA, ssGSEA, NTP and Ingenuity Pathway Analyses (IPA). All gene signatures used are available in Molecular Signature Database (MSigDB, Broad Institute) or were previously reported (**Supplementary Table 2**). CIBERSORT[25] (cell-type identification by estimating relative subsets of RNA transcripts) was used to estimate the relative fraction of 22 immune cell types within the leukocyte compartment of non-tumor liver tissues. The Immunophenoscore (IPS) algorithm[29] was used to analyze the major immunogenic determinants. An *in silico* analysis based on ssGSEA scores of ~1230 gene sets (DSigDB) recapitulating targets of approved therapies was also performed for the screening of candidate targeted therapies. This approach provided the rationale for testing nintedanib in a preclinical model of HCC development.

Histological evaluation of infiltrating inflammation

Histopathological analysis was performed in 98 out of 167 cases. Specifically, hematoxylin and eosin (H&E) staining of formalin-fixed paraffin embedded (FFPE) tissue section of HCCs and their matched adjacent non-tumor livers were evaluated by two expert pathologists (CM and MS). The presence of inflammation (portal/septal, interface, pericentral and lobular) as well as the lymphoid aggregates were assessed in the non-tumor liver tissue sections. More details on the histological evaluation of the samples have been included in **Supplementary material**.

Animal model

For the purpose of this study, we generated a chemically-induced model of HCC and fibrosis in male C57BL/6J mice (Harlan Laboratories, n=58) by a single injection of N-nitrosodiethylamine (DEN) followed by weekly dosing with carbon tetrachloride (CCl_4), as previously described[30]. Once fibrosis was established, mice were randomized to receive vehicle or nintedanib (50 mg/kg, Boehringer Ingelheim). Mice were then sacrificed at different time-points and liver and tumor tissue samples were collected and processed for molecular characterization of histology, the transcriptome and protein expression. All experimental procedures were carried out following the approval of the institutional ethical committee of the University of Barcelona and Hospital Clinic of Barcelona. For specific details, see **Supplementary material**.

Statistical analysis

All analyses were performed using SPSS software version 23 (IBM) or GraphPad Prism version 5.00 (San Diego, CA). Correlations for categorical and continuous variables were analyzed by Fisher's exact test and Wilcoxon rank-sum test, respectively. The prognostic value of the signatures was assessed using Kaplan-Meier estimates, log-rank test, and Cox regression models. In *in vivo* studies, the Mann-Whitney U test was used to compare differences in body weights, liver function, tumor number, and tumor size. Fisher exact test was performed for analysis of HCC incidence and pERK staining. Student T-Test was used to compare the differences in Sirius Red quantification, CD31 staining, CD4/CD8 staining proportion of immune cell infiltrate and relative gene expression.

RESULTS

Identification of a novel immune-mediated cancer field effect in non-tumor liver tissue of patients with early HCC.

In order to characterize the immune features governing the unresolved cancer-field in which new cancers arise, transcriptome-based analysis of a compendium of ~5,000 annotated immunology-specific gene-sets[23] was performed in the non-tumor liver tissue of patients with early stage HCC. This analysis revealed the presence of an immune-mediated cancer field (ICF) in ~60% (98/167) of samples (**Figure 1A and Supplementary Figure 1**). Specifically, these samples were characterized by enrichment of several gene-sets recapitulating the presence of activated immune cells, up-regulation of core signaling pathways involved in immune response (both innate and adaptive) as well as those involved in the modulation of inflammatory response (i.e. IL2-STAT5, IL6-STAT3, IL17, IFN-γ, CSF, TNF-α, and TGF-β signaling) (**Figure 1A-B**

and **Supplementary Figure 1**). Moreover, histological evaluation confirmed that liver tissues with ICF contained a higher frequency of moderate to marked inflammatory infiltrate (74% in ICF vs. 52% in non-ICF, p=0.034) and lymphoid aggregates (80% in ICF and vs. 55% in non-ICF, p=0.009) (**Figure 1C-1D and Supplementary Table 3**). In contrast, histological evaluation of the tumor showed no significant correlation between the presence of the ICF and the detection of intratumoral or peritumoral infiltration (**Supplementary Table 3**). This is in accordance with our recent publication[20], where the tumor immune-based profile did not correlate with presence or absence of immune gene signatures in the surrounding non-tumor tissue.

While characterizing the ICF we detected that, in addition to immunogenic features, several well-known pro-carcinogenic signals such as epithelial-to-mesenchymal transition, KRAS, EGFR, and VEGF signaling were also significantly enriched in liver tissues containing the ICF (**Supplementary Table 4**). In line with these oncogenic signals, a significant enrichment of previously reported prognostic signatures derived from the adjacent non-tumoral liver were also detected. These signatures included the 186-gene cancer-field signature[13], activated hepatic stellate cells (HSCs)[17], hepatic injury and regeneration (HIR)[19], and multicentric occurrence of HCCs[31] (**Figure 1A**). The presence of the ICF significantly correlated with HCV infection, poor survival [median OS 43.4 mo in the ICF group vs 94.8 mo in non-ICF; p=0.001] and features indicative of liver dysfunction such as high bilirubin, low platelet count and albumin levels (**Supplementary Table 5 and Supplementary Figure 1B**). Altogether, our data highlight the presence of an immune-mediated cancer field in 60% of early HCC patients. This ICF is characterized by activation of immunomodulatory signaling cascades (i.e. IFN-γ, TNF-α, TGF-β, IL6) along with cancer promoting signaling pathways (i.e. EMT, EGFR and VEGFR), and is associated with HCV infection and poor prognosis.

The immune-mediated cancer field contains 3 distinct molecular subtypes.

Further dissection of the key immune-modulating signaling pathways and immune-cell infiltrates in those samples harboring the immune-mediated cancer field revealed the existence of three distinct molecular subtypes. The first molecular subtype, henceforth called the "*High Infiltrate-ICF*" subtype (23% of the ICF), showed a significant enrichment of several previously established gene signatures mirroring the presence and/or activation of immune cell infiltrates such as lymphocytes (T and B cells)[27,32], macrophages[33] and ectopic lymphoid structures[34] (**Figure 2A-2B**). Consistently, immunogenicity, herein captured either by the cytolytic activity score (**Figure 2A**)[35] or

using the immunophenoscore algorithm[29] (**Figure 2C**), was also significantly higher in these samples (p<0.001). Specifically, non-tumor liver samples belonging to the *High Infiltrate-ICF* subtype showed significant infiltration of effector cells, including activated and memory CD8+/CD4+ T cells (p<0.01) together with up-regulation of the main components of the major histocompatibility complex (MHC) class I and class II (**Figure 2C,** p≤0.001). The second subtype, the so-called "*Immunosuppressive-ICF*" (36% of the ICF), was characterized primarily by activation of stroma and HSCs, increased TGF-β signaling and T cell exhaustion (**Figure 2A**). Moreover, several immune-checkpoint inhibitors (i.e. *CTLA-4, TIGIT, LAG*3) were significantly over-expressed (p<0.01) in this class, along with higher levels of M2 macrophages (p<0.05), activated mast cells (p<0.05), and neutrophils (p<0.01) which are among the main mediators of immune tolerance and inhibition (**Figure 2B-2C**). The third subtype (41% of the ICF) showed a clear predominance of IFN-γ signaling (p<0.001) and enrichment of the inflammatory M1 macrophages (p<0.001) and follicular helper T cells (p<0.05), and was called the "*Pro-inflammatory-ICF*" subtype (**Figure 2**). Interestingly, the *High Infiltrate* and *Immunosuppressive* subtypes shared several molecular features including the enrichment of key signaling pathways involved in modulating the immune response (i.e. IL2 and TNF signaling), proliferation (i.e. KRAS signaling) and angiogenesis (**Figure 2A,** p<0.001).

In order to further confirm the presence and molecular traits of the identified ICF, we generated a transcriptome-based gene signature able to capture the three immune-mediated cancer field subtypes. Interestingly, this signature only showed minimal overlap (0-5%) with previously reported gene signatures of field cancerization in HCC (**Supplementary Table 6**) [13,16,18,36]. The predictive capacity of the resulting 172-gene signature was then validated in the adjacent non-tumor tissue of 225 patients with early HCC, previously characterized by our group[13,37] (**Supplementary Figure 2, Supplementary Table 7**). Similar to what previously observed in the training cohort, 58% (130/225) of patients belonged to the immune-mediated field. Within this group, ~31% (40/130) presented the *High Infiltrate-ICF* profile, ~27% (35/130) the *Immunosuppressive-ICF* and ~42% (55/130) the *Pro-inflammatory-ICF* subtype (**Supplementary Figure 2**). Subsequent characterization further confirmed the ability of the signature to capture the main molecular traits defining each subtype, such as increased infiltration of effector T cells in *High Infiltrate* subtype, activation of stroma and TGF-β signaling in *Immunosuppressive* subtype and up-regulation of IFN-γ signaling in *Pro-inflammatory* subtype (**Supplementary Figure 2**). Overall, our results highlight the presence of 3 molecular subtypes within the immune-mediated cancer

field with high degree of lymphocyte infiltration (overall 16% of HCC patients) or either immunosuppressive (overall 20% of HCC patients) or pro-inflammatory (24% of HCC patients) signaling cascades.

The immunosuppressive-ICF subtype predicts high risk of HCC development in cirrhotic patients

Following the identification of an immune-mediated cancer field in the livers of 60% of patients with early HCC, we next sought to assess its role in liver disease progression and HCC primary occurrence. To this end, the 172-gene signature was analyzed in a cohort of 216 non-malignant cirrhotic patients with a median follow-up of 10 years in the context of an HCC surveillance program[16]. Overall, 51% (110/216) of cirrhotic patients harbored the ICF, including the *High Infiltrate-ICF* subtype in 28% (31/110), the *Immunosuppressive-ICF* subtype in 19% (21/110), and the *Pro-inflammatory-ICF* subtype in 53% (58/110) of cirrhotic patients harboring the ICF. Next, we tested the capacity of the ICF subtypes to predict the risk of HCC development in cirrhotic patients. Interestingly, the presence of the *Immunosuppressive-ICF* subtype (10% of all cirrhotic patients) was significantly associated with a higher risk of HCC development [median time to HCC development of 7.4 years (95% CI: 3.2-11.7) *vs* 17.1 years (95% CI: 10.6-23.7) in Rest, p<0.0001] and was found to be an independent predictor of HCC occurrence in cirrhotic patients in a multivariate analysis [HR 2.41 (95% CI: 1.2-4.8), p=0.012] (**Figure 3A** and **Table 1**). In addition, the *Immunosuppressive-ICF* was also significantly associated with poor survival [median overall survival of 7.1 years (95% CI: 4.5-9.6) *vs* 16.3 years (95% CI: 9.1-23.5) in Rest, p<0.0001] and higher risk of hepatic decompensation [median time to hepatic decompensation of 6.5 years (95% CI: 4.3-8.6) *vs* >15 years in Rest, p<0.0001] (**Figure 3B-3C**). Cirrhotic patients harboring the other two ICF subtypes (*High Infiltrate* and *Pro-inflammatory* subtypes) also showed a non-significant trend towards a higher risk of HCC development compared to those patients lacking the ICF [mean time to HCC development of 12.8 years (95% CI:11.5-14.2) in Other-ICF subtypes *vs* 16.3 years (95% CI: 14.2-18.5) in non-ICF, p=0.06] (**Supplementary Figure 3A**).

Moreover, the analysis of an additional cohort of 124 non-neoplastic patients with liver fibrosis[22] revealed that the immune-mediated cancer field may occur as a progressive event, as it significantly correlated with increasing levels of fibrosis stage and degree of inflammation (**Supplementary Figure 3B**). Particularly, the presence of the

Immunosuppressive-ICF significantly correlated with the presence of advanced liver fibrosis (Scheuer fibrosis S3-4 score[22], p=0.034) (**Supplementary Figure 3B**).

In conclusion, the immune-mediated cancer field detected in patients with early HCC is also present in the livers of ~50% of cirrhotic patients and captures the presence of a damaging and continuous inflammatory response in the underlying liver disease. Furthermore, our results underscore the critical role of an *Immunosuppressive-ICF* cancer field (overall, 10% of cirrhotic patients) in defining a 2.4 risk of HCC development vs the rest of patients, and to a smaller extent of the *High Infiltrate* and *Pro-inflammatory* subtypes.

The immune-mediated field as target for chemoprevention in a mouse model recapitulating chronic liver inflammation and HCC development

Based on the compelling results described above, we hypothesized that the immune-mediated cancer field, and particularly the *Immunosuppressive-ICF* subtype, may represent an ideal target for chemopreventive strategies in cirrhotic patients at high risk of HCC development. To this purpose, an *in silico*-based analysis was performed using our training cohort to identify those candidate therapies most likely to modulate the identified ICF. This analysis was based on the enrichment of a compendium of ~1230 gene sets (DSigDB collections D1 and D2)[38] recapitulating the main targets of 1202 approved drugs. Among the top 10 most significantly enriched drugs **(Supplementary Figure 4**), nintedanib was the only FDA-approved therapy indicated for a non-cancer condition. Specifically, nintedanib is the first molecular targeted therapy with clinical efficacy in patients with idiopathic pulmonary fibrosis as both an anti-fibrogenic and anti-inflammatory agent[39]. Given these considerations, the efficacy of nintedanib in reverting the pro-tumorigenic immune-mediated cancer field was tested in a mouse model of HCC development in the setting of chronic inflammation and liver fibrosis (**Supplementary Figure 5A**). In this model, the macroscopic evaluation of explanted livers in DEN/CCl_4 treated mice sacrificed at the age of 15, 17 and 18 weeks confirmed the development of numerous hepatic tumors in the context of chronic inflammation and liver fibrosis (**Figure 4A**). Tumor penetrance and number of tumors progressively increased, ultimately reaching a 100% incidence at 18 weeks of age (**Figure 4A and 4B**). At all-time points, histological evaluation of the liver sections showed that a portion of the tumors were pre-neoplastic (dysplastic) nodules (**Figure 4C**). After only 3 weeks of treatment (mice sacrificed at 15 weeks of age, **Supplementary Figure 5A**), nintedanib showed a clear trend towards reducing HCC incidence, number and size of

tumors (**Figure 4B, D and E**). These differences reached significance at 17 weeks of age (**Figure 4B**), having a marked decrease in both overall number of tumors (30% in nintedanib vs 89% in vehicle group, p=0.019) and specifically in HCC incidence (7% in nintedanib vs 33% in vehicle group, p=0.04). Similarly, at 18 weeks of age, HCC incidence was significantly reduced in treated mice (**Figure 4B**, 22% in nintedanib vs 77% in vehicle group, p<0.001). In addition, nintedanib significantly reduced the overall tumor number and size both at 17 and 18 weeks of age (**Figure 4D-E**). Overall, nintedanib was well tolerated with no significant induction of body weight loss or hepatotoxicity measured by serum ALT and AST levels (**Supplementary Figure 5B-C**). Taken together, our data suggest that nintedanib is safe and efficacious in preventing HCC development in our experimental model.

Nintedanib treatment reverts the immunosuppressive-ICF effect

Next, we sought to assess the impact of nintedanib treatment on the immune-mediated cancer field. For this purpose, we analyzed gene expression profiling of non-tumor liver samples from 17 weeks-old DEN/CCl_4 mice treated with nintedanib (n= 10) or vehicle (n=9), and 3 healthy control mice. First, the comparison between the healthy control group and vehicle treated mice revealed a profile of activated pathways compatible with HCC development within a fibrotic and inflammatory background. In this regard, functional analysis of differentially expressed genes (**Supplementary Table 8**) highlighted the activation of hepatic stellate cells and fibrogenesis, as well as immune system activation (inflammatory response, chemotaxis, binding of myeloid and leukocytes) in vehicle treated DEN/CCl_4 livers (**Supplementary Table 9**). Notably, our model faithfully recapitulated the human immune-mediated field subtypes described above (**Figure 5A**)**.** The comparison of the gene expression profiles of adjacent non-tumor liver from mice treated with vehicle or nintedanib demonstrated that nintedanib significantly reverted the different ICF subtypes, more specifically the *Immunosuppressive-ICF* phenotype, which predicts risk of HCC development in cirrhotic patients (**Figure 5A,** p=0.005). A non-significant trend was also observed for the other two types of ICF (**Figure 5A**). Treatment with nintedanib led to a significant down-regulation of inflammatory cues (IL-6/STAT3, interferon-α, interferon-γ) and immune-related signaling (IL-2/STAT5 activation, allograft rejection) **(Figure 5A).** Among the infiltrating immune cells, nintedanib significantly reduced the presence of B and T cells, activated macrophages, helper T cells and Tregs (**Figure 5A**). Furthermore, nintedanib significantly decreased the expression levels of immune response modulators characteristic of the *Immunosuppressive-ICF* subtype (including

IL1, CCL5, *PDL1*, **Figure 5A**). Despite exhibiting similar global levels of inflammatory infiltrates, quantification of CD4 and CD8 positive infiltrating lymphocytes by IHC revealed a significant decrease of CD4+ T cells in nintedanib-treated mice compared to controls (**Figure 5B**, $p<0.05$). These data are in accordance with the down-regulation of gene sets representing helper and regulatory T cells (**Figure 5A**).

Next, in order to further characterize the chemopreventive effects of nintedanib we assessed the activation status of the main nintedanib targets (i.e. VEGFR2 and PDGFR-β). Western blot of non-tumor liver tissue confirmed that nintedanib blocked the activation of VEGFR2 (**Figure 5C**) and its downstream effectors AKT and ERK (**Supplementary Figure 6A**)**.** Consistently, both liver parenchyma and liver tumors were pERK positive in vehicle-treated mice and pERK negative in nintedanib treated mice (**Supplementary Figure 6B**, $p<0.05$), indicating an anti-proliferative effect of nintedanib as well. Given the strong inhibition of VEGFR signaling observed, we next assessed the anti-angiogenic effect of nintedanib in DEN/CCl_4 mice. In this model, reduced CD31 staining was associated with diminished blood vessel area in both liver parenchyma and liver tumors of nintedanib-treated mice (**Figure 5D**). Altogether, these data suggest that nintedanib exhibits its chemopreventive effects in part by inducing vascular normalization and inhibiting hepatic proliferation.

In parallel, we evaluated the potential of nintedanib to ameliorate liver fibrosis. No significant inhibition of the main pro-fibrogenic signaling pathway**,** PDGFR-β, was detected in the livers of nintedanib-treated mice (**Supplementary Figure 6C**). Consistently, similar levels of fibrosis degree were observed between mice treated with nintedanib or vehicle (**Supplementary Figure 6D**). Furthermore, the gene expression levels of collagen markers (i.e. *Col1a1* and *Col1a2*) and the pro-fibrogenic ligand *Pdgfb* were similar in nintedanib- and vehicle-treated mice (**Supplementary Figure 6E**). In contrast, α-SMA (Acta2), a marker of extracellular matrix (ECM) producing cells, was significantly decreased in nintedanib-treated mice at 17 weeks of age, with a non-significant trend towards reduction at 18 weeks (**Supplementary Figure 6E**), in accordance with the reduced liver angiogenesis.

Overall, our data confirm that our mouse model faithfully recapitulates the immune-mediated cancer field effect observed in human samples and that therapeutic targeting of the *Immunosuppressive-ICF* subtype, accompanied by liver vascular normalization and suppression of hepatic proliferation, can prevent the development of HCC associated with advanced chronic liver disease.

DISCUSSION

This study represents an in-depth analysis of the *inflammatory milieu* associated with the "field cancerization" in the chronically injured liver, and investigates its clinical implications in the prediction and prevention of HCC occurrence in cirrhotic patients.

The role of the "cancer field effect" in promoting neoplastic transformation has gained much interest in recent years and currently an altered microenvironment is considered a promoter of cancer[40,41]. Activation of HSC as well as certain pathways, such as nuclear factor-KB and TGF-β signaling, have been previously associated with liver fibrogenesis, and eventually carcinogenesis[13,18]. With this study, we move beyond the limits of current knowledge and provide a detailed description of the immune microenvironment underlying the *field cancerization* in the non-tumor liver. To this end, we first characterized the immune profile of the non-tumor liver parenchyma of 392 early HCCs and then investigated its role in predicting HCC development in 214 cirrhotic patients with long-term surveillance for HCC (median of 10 years)[16]. The analysis revealed that up to 60% of HCCs and 50% of cirrhotic patients showed a deleterious immune-mediated response in the surrounding tissue, which was associated with impaired liver function, activation of specific oncogenic loops and poor survival. Further dissection of the immunological portrait of this molecular group identified three distinct subtypes with different levels of lymphocyte infiltration and activation of either *immunosuppressive* or *pro-inflammatory* traits were identified. Interestingly, the *Immunosuppressive-ICF* subtype (~10% of cirrhotic patients), mostly characterized by stromal activation and TGF-β signaling, was an independent predictor of HCC development, increasing 2.4 the risk of cancer development on top of the other cirrhotic molecular subtypes. These observations support the hypothesis that an immunosuppressive microenvironment favors HCC development and provide insights into the immune-mediated mechanisms underlying the cancerization field. Perhaps of greater clinical relevance, the *Immunosuppressive-ICF subtype* may provide a novel companion biomarker to enrich at-risk patients in chemoprevention clinical trials.

Reducing the incidence and mortality of HCC requires advances in chemopreventive approaches at pre-neoplastic stages, in addition to curative treatment options for early lesions. Universal immunization against HBV and antiviral therapies against HBV and HCV have been associated with very reduced HCC risk[2,42–44]. Once cirrhosis is established, the risk of HCC development remains despite achieving a sustained virologic response in HCV patients[11,12]. In addition, the incidence of other risk factors, such as non-alcoholic steatohepatitis (NASH), is dramatically increasing[45]. Thus, alternative HCC preventive strategies capable of interfering with molecular

hepatocarcinogenesis are an unmet need. Furthermore, identifying those patients at high risk of HCC development should enable a cost-effective selection of patients most likely to benefit from chemopreventive approaches. Since our results specifically showed that the *Immunosuppressive-ICF* class identifies 10% of cirrhotic patients at higher risk of HCC development (20% in patients that already developed an HCC), we then sought to investigate if the molecular forces driving such cancer field could serve as target for chemopreventive strategies. To test this hypothesis in the preclinical setting, we first verified that the molecular profiles observed in human cirrhosis were faithfully reproduced in animal models of chronic liver injury. The DEN/CCl_4 chemically-induced mouse model reliably reproduced the presence of a pro-carcinogenic phenotype with increased inflammation, angiogenesis and immune response. In particular, characterization of the immune-mediated field recreated in those animal livers confirmed the recruitment of T cells, regulatory and helper T cells observed in liver tissues from patients belonging to the immune-mediated cancer field.

In order to identify the most promising candidate therapies for novel chemopreventive strategies, we conducted an *in silico* analysis using a large compendium of gene sets[38] recapitulating the main targets of 1202 approved drugs. Among the top ten most significantly enriched drugs, we selected nintedanib, the only FDA therapy approved for non-neoplastic conditions. In our animal model, oral administration of nintedanib reverted the immune-mediated cancer field, and particularly the *Immunosuppressive-ICF* subtype, ultimately reducing HCC incidence and growth. Reversion of the *Immunosuppressive* profile induced by treatment with nintedanib was accompanied by reduction of CD4+ lymphocytes. These findings are in line with previous reports suggesting that CD4+, but not CD8+ T cells, propagate immune-mediated liver injury in models of chronic liver inflammation or autoimmune liver disease[46,47]. Pretreatment with T cell-specific Abs or immunosuppressive agents, such as anti-CD4 mAb, FK506 (Tacrolimus), or cyclosporine A, have shown to ameliorate hepatitis in these models, further supporting the role of CD4+ T cells in inducing liver damage[48]. The damage is thought to be partially mediated by T-cell-derived IFN-γ and Kupffer-cell-derived TNF, which lead to hepatocyte cell death[49]. Consistently, elevated IFN-γ and TNF signaling were among the key features up-regulated in our immune-mediated cancer field and were both significantly inhibited upon treatment. Overall, our study identifies a novel promising chemopreventive strategy for HCC and confirms the validity of using the reversion of the *Immunosuppressive-ICF signature* as reliable read-out of efficacy. This is of great clinical importance since there is currently no effective method to monitor the short-term effects of chemopreventive drugs[10].

Angiogenesis and inflammation are interdependent and, as demonstrated in different studies conducted in animal models and human tissues, are active partners at onset of cancer[50]. Thus, blockage of angiogenesis can also represent an attractive chemoprevention strategy[51]. Nintedanib belongs to a new generation of TKIs that, in addition to exerting immune modulating by inhibiting src family of kinases (i.e. LCK, FLT3,and SRC), blocks the activation of main angiogenic receptors[52]. Many cytokines and growth factors are involved in modulating the formation of new vessels. Expression of *VEGF* and its receptors is elevated in HCC cell lines and tissues, as well as in the blood circulation of patients with HCC[37,53–55]. In our model, nintedanib exerted its chemopreventive mechanisms in part through the inhibition of VEGF signaling, a major driver of angiogenesis[56]. Consistently, nintedanib treatment led to significant decrease of α-SMA and CD31, markers highly expressed on activated endothelial cells. Thus far, independent studies had described that HCC prevention can be achieved in animal models by attenuating liver fibrosis through the inhibition of epidermal growth factor receptor (EGFR)[57,58] or lysophosphatidic acid (LPA)[36] signaling. With the current study, we demonstrate that modulation of the liver microenvironment by molecular targeted drugs, which simultaneously block liver inflammation and angiogenesis, might represent a powerful alternative strategy .

We recently defined the immune class of HCC[20] and the Immune exclusion class (characterized by active Wnt/CTNNB1)[3,20,59], which might predict response and primary resistance to checkpoint inhibitors, respectively [3,59]. We herein explore the immune-mediated mechanisms underlying HCC occurrence by defining an immunosuppressive field effect in ~10% of cirrhotic patients that conforms a cancer-permissive *milieu*, thus posing them at the highest risk of HCC development. In addition, our pre-clinical data with a drug approved in pulmonology and in non-small cell lung cancer treatment suggest that the permissive microenvironment can be reverted leading to a delay in HCC occurrence. These data provide the rationale for testing this strategy in early chemoprevention trials targeting cirrhotic patients at high risk of HCC development. In addition, this strategy could also be further explored in the adjuvant setting considering that 20% of HCC undergoing resection also present this permissive *milieu* in the adjacent non-tumoral tissue.

FIGURE LEGEND

Figure 1. Identification of an immune-mediated cancer field effect in non-tumoral liver tissue adjacent to early HCCs. A) Heatmap representation of the immune-mediate cancer field present in 60% of HCC patients. High and low single sample gene-set enrichment (ssGSEA) analysis scores are represented in red and blue, respectively. Positivity for previously reported gene signatures was evaluated by nearest template (NTP) method. B) Top predicted upstream cytokine and transcription factors activated in liver tissues belonging to the immune-mediated field effects. C) Representative images of degree of Portal/Septal infiltrating inflammation. Moderate-to-marked inflammatory infiltrate was more prevalent in samples with immune-mediated field effect. D) Representative images depicting presence or absence of lymphoid aggregates. The presence of lymphoid aggregates was significantly associated with the immune-mediated cancer field effect.

Figure 2. The immune-mediated cancer field contains 3 distinct molecular subtypes. A) Heatmap representation of the three ICF subtypes within the samples presenting the immune-mediated cancer field. Statistical significance is highlighted comparing the different subtypes within the ICF (*Purple* in High Infiltrate-ICF, *Orange* in Immunosupressive-ICF, *Green* in Pro-inflammatory-ICF, and *Black* in both High Infiltrate and Immunosuppressive). B) Comparison of estimated proportion of immune cells (CIBERSORT method) between the immune-mediated cancer field subtypes. C) Immunophenogram representing the enrichment of immunogenic determinants in the distinct immune-mediated cancer field subtypes. Significant statistical differences are defined as follows: *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$.

Figure 3. Association of the presence of the *Immunnosuppressive-ICF* with HCC occurrence and prognostic variables in cirrhotic patients. (A) Kaplan-Meier estimates of HCC development, (B) overall survival, (C) hepatic decompensation, according to the presence of the *Immunosuppressive* -ICF subtype (orange), and the Rest of the cohort.

Figure 4. Nintedanib prevents and delays the development of HCC. A) Representative pictures of macroscopic evaluation of hepatic tumors in mice treated with vehicle or nintedanib sacrificed at 15, 17 and 18 weeks of age. Arrows indicate macroscopically visible tumors. B) Evaluation of overall tumor burden and HCC

incidence. ($^{\#}$) denotes statistical significance for overall tumor burden while (*) for HCC incidence. C) Microscopic evaluation of the number of tumors per mouse in each experimental group. Ill-defined hepatic tumors were diagnosed as dysplastic nodules, while circular well-defined lesions with pushing margins and/or vascular invasion were diagnosed as HCCs. D) Number of macroscopic tumors per mouse treated with vehicle or nintedanib at the three different time-points. E) Diameter size of the largest tumor per mouse treated with vehicle or nintedanib at the three different time-points. A total of 7-11 mice were evaluated per group in each approach. Significant statistical differences are defined as follows: $^{\#}$ or *=p<0.05, **=p<0.01 and ***=p<0.001.

Figure 5. Nintedanib reverts the *Immunosuppressive-ICF* recapitulated in the DEN/CCl_4 mice and induces vascular normalization. A) Single sample GSEA analysis of the 172-gene signature and gene-sets recapitulating the immune-mediated cancer field subtypes. In the heatmap, high and low ssGSEA analysis scores or gene expression levels are represented in red and blue, respectively. B) Representative images and quantification of CD4+ and CD8+ positive infiltrating lymphocytes in the liver parenchyma of 17 weeks old mice treated with vehicle (n=5) or nintedanib (n=5). C) Western-blot analysis of VEGFR2 activation in the non-tumor liver parenchyma of 17 weeks old mice treated with vehicle (n=6) or nintedanib (n=6). D) Morphometric quantification of blood vessel area by CD31 immunostaining in 5 randomly selected low magnification fields in mice treated with vehicle (n=5) or nintedanib (n=5). Significant statistical differences are defined as follows: **=p<0.01 and ***=p<0.001

TABLES

Variable	Univariate analysis p-value	Multivariate analysis (cox's regression) HR	CI(95% low-high limits)	p-values
Non-tumoral liver tissue-based transcriptomic profiles				
Immunosuppressive-ICF	**0.03**	**2.41**	**1.21-4.80**	**0.01**
186-gene Poor prognosis signature	**<0.0001**	1.56	0.89-2.7	0.12
Clinicopathological variables				
Age (>median)	0.87			
Gender	0.22			
Diabetes	0.48			
HCV genotype 1b	0.18			
Alcohol consumption (>80 g/day)	0.68			
HCVetiology plus alcohol consumption	0.68			
History of antiviral treatment (interferon-based)	0.65			
Varices	**0.02**	1.49	0.85-2.6	0.17
Spleen	0.13			
Ishaak score 6 vs 5	0.24			
Platelet count (<100,000/mm3)	**0.02**	1.51	0.91-2.64	0.15
Bilirubin (> 1 mg/dL)	**0.00**	**1.85**	**1.07-3.2**	**0.03**
AFP (> 10 ng/mL)	0.87			
Prothrombin time (international normalized ratio >1.2)	0.38			

Table 1. Uni- and Multivariate Analysis of risk of HCC development in cirrhotic patients including gene signatures and clinico-pathological variables (n=216).

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