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Identification of an Immune-specific Class of Hepatocellular Carcinoma, Based on Molecular Features

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BACKGROUND & AIMS: Agents that induce an immune response against tumors by altering T-cell regulation have increased survival times of patients with advanced-stage tumors, such as melanoma or lung cancer. We aimed to characterize molecular features of immune cells that infiltrate hepatocellular carcinomas (HCCs) to determine whether these types of agents might be effective against liver tumors. **METHODS:** We analyzed HCC samples from 956 patients. We separated gene expression profiles from tumor, stromal, and immune cells using a non-negative matrix factorization algorithm. We then analyzed the gene expression pattern of inflammatory cells in HCC tumor samples. We correlated expression patterns with the presence of immune cell infiltrates and immune regulatory molecules, determined by pathology and immunohistochemical analyses, in a training set of 228 HCC samples. We validated the correlation in a validation set of 728 tumor samples. Using data from 190 tumors in the Cancer Genome Atlas, we correlated immune cell gene expression profiles with numbers of chromosomal aberrations (based on single-nucleotide polymorphism array) and mutations (exome sequence data). **RESULTS:** We found approximately 25% of HCCs to have markers of an inflammatory response, with high expression levels of the CD274 molecule (programmed death-ligand 1) and programmed cell death 1, markers of cytolytic activity, and fewer chromosomal aberrations. We called this group of tumors the Immune class. It contained 2 subtypes, characterized by markers of an adaptive T-cell response or exhausted immune response. The exhausted immune response subclass expressed many genes regulated by transforming growth factor beta 1 that mediate immunosuppression. We did not observe any differences in numbers of mutations or expression of tumor antigens between the immune-specific class and other HCCs. **CONCLUSIONS:** In an analysis of HCC samples from 956 patients, we found almost 25% to express markers of an inflammatory response. We identified 2 subclasses, characterized by adaptive or exhausted immune responses. These findings indicate that some HCCs might be susceptible to therapeutic agents designed to block the regulatory pathways in T cells, such as programmed death-ligand 1, programmed cell death 1, or transforming growth factor beta 1 inhibitors.

Keywords: Immune Checkpoint; Virtual Microdissection; Molecular Subgroups; Immune Regulation.

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related mortality worldwide. The number of HCC deaths (approximately 800,000 per year) overlap with that of new cases, a testament to its high lethality.[1,2] This malignancy often occurs in the setting of chronic inflammatory liver disease (eg, cirrhosis) and is associated with well-defined risk factors such as hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol abuse, metabolic syndrome, and diabetes.[2] Over the past decade, major advancements have elucidated the molecular pathogenesis of HCC,[2,3] and yet, current therapeutic options remain very limited. Only a minority of patients with HCC are diagnosed at early stages when curative approaches, such as surgical resection, transplantation, or local ablation, are effective.[2] In patients at advanced stages, the only systemic therapies that increase survival are the multi–tyrosine kinase inhibitors sorafenib (first line)[4] and regorafenib (second line).[5]

Abbreviations used in this paper: **CCL, chemokine (C-C motif) ligand; CTNNB1, catenin beta 1; CXCL, chemokine (C-X-C motif) ligand; FDR, false discovery rate; FF, fresh frozen; FFPE, formalin-fixed paraffin-embedded; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; LGALS, lectin; galactose binding, soluble 1; NK, natural killer; NMF, non-negative matrix factorization; NTP, nearest template prediction; PD-1, programmed death 1; PD-L1, programmed death-ligand 1; PTK2, Protein Tyrosine Kinase 2; SCNA, somatic copy number aberrations; TCGA, The Cancer Genome Atlas; TGF-β, transforming growth factor beta; TLS, tertiary lymphoid structure.**

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Nonetheless, even with the survival benefits provided by these agents, the median life expectancy is less than 2 years. Therefore, there is a clear need to expand the therapeutic arsenal for advanced HCC.

In recent years, immune checkpoint inhibitors, which unleash the body's own immune response to attack tumors by targeting regulatory pathways in T cells, have shown remarkable efficacy in different solid cancers; this has led to the Food and Drug Administration approval of 4 immune-based compounds for the treatment of advanced-stage malignancies, such as melanoma or lung cancer (ie, ipilimumab, nivolumab, pembrolizumab, and atezolimumab). These agents include monoclonal antibodies directed against the cytotoxic T-lymphocyte protein 4, the programmed cell death protein 1 (PD-1) and its ligand PD-L1.[6] Intriguingly, not all patients have the same likelihood of responding to these regimens.[7] High expression of PD-L1 is currently under investigation as a potential predictor of response to anti-PD1 therapy.[8–10] Emerging experimental data indicate that the presence of a preexisting intratumoral T-cell infiltration, interferon (IFN) signaling, checkpoint molecules (PD-1, PD-L1 expression) or high tumor mutational burden could favor a clinical response.[11–13] Conversely, tumor-intrinsic active ß-catenin (CTNNB1) signaling may result in T-cell exclusion and resistance to anti–PD-L1 and anticytotoxic T-lymphocyte protein 4 antibodies.[14] In HCC, promising responses have been recently reported with nivolumab, a monoclonal antibody directed against PD-1, in a phase I/II trial.[15] Unfortunately, little is known about the immunological profile of HCC tumors and how to leverage this information to maximize response to immune-based therapies.

HCCs comprise a mixture of cell types, including malignant hepatocytes, immune cells, and endothelial cells dispersed within the extracellular matrix and supporting stroma. Previous studies have established a set of analytical approaches to virtually dissect the molecular signals deriving from these distinct compartments.[16,17] Using non-negative matrix factorization (NMF), we have deconvoluted the gene expression data of 956 human HCC samples and isolated the signal released from the inflammatory infiltrates to characterize the immunologic landscape of HCC. This has allowed us to identify an immune-specific class of HCC with specific biological traits. Key features of this class include actual presence and activation of immune cells, enhanced cytolytic activity, protein expression of PD-1 and PD-L1, and enrichment of gene signatures predictive of response to immunotherapies. Further dissection of this class has revealed 2 robust microenvironment-based types with either active or exhausted immune activity. These findings provide a comprehensive understanding of the immunologic milieu of HCC and deserve further investigation in patients with HCC treated with immunotherapy.

Materials and Methods

Patients and Samples

For the purpose of the study, gene expression profile from a total of 956 HCC human samples was analyzed (Figure 1), including a training cohort of 228 surgically resected fresh frozen (FF) samples (Heptromic dataset, GSE63898). All samples of the training set were previously obtained from 2 institutions of the HCC Genomic Consortium on institutional review board approval: IRCCS Istituto Nazionale Tumori (Milan, Italy) and Hospital Clínic (Barcelona, Spain). RNA profiling and methylation data were available for all 228 HCC samples and 168 nontumor liver adjacent cirrhotic tissues and are published elsewhere.[18] An additional 728 HCC samples of patients with mixed etiology from 7 independent datasets were used for external validation (Figure 1, Supplementary Table 1).

Statistical Analysis

All analyses were performed using SPSS software version 22 (IBM Corporation, Chicago, IL). Correlations among molecular classes, histologic markers, and clinico-pathologic variables were analyzed by Fisher's exact test and Wilcoxon rank-sum test for categorical and continuous data, respectively. All signatures used in the study were previously reported (Supplementary Table 2).

Additional detailed protocols are provided in the Supplementary Materials and Methods.

Results

A Novel Immune Class of HCC

To isolate immune-related genomic signals from bulk gene expression data in HCC tumors, we performed NMF analysis of 228 resected HCC samples (training cohort, Figure 1). Clinical characteristics of the training cohort are summarized in Table 1. Among the distinct expression patterns identified by NMF, one was attributed to the presence of inflammatory response and immune cells through integration with a previously reported immune enrichment score (Supplementary Figure 1*A*). Analysis of the top-ranked genes (named exemplar genes) that defined this expression pattern further confirmed immune-related functions and signaling (Supplementary Figure 1*B*). Consensus clustering on exemplar genes (Supplementary Figure 2) identified a new molecular subgroup accounting for 24% of the cohort (55/228), referred herein as "Immune class" (Figure 2*A*). Patients belonging to the Immune class showed significant enrichment of signatures identifying immune cells (ie, T cells, cytotox, tertiary lymphoid structures [TLS], and macrophages [$P < .001$]), immune metagenes, IFN gene signatures predictive of response to pembrolizumab in melanoma (28 genes, $P < .001$), and head and neck squamous cell carcinoma (6 genes, $P < .001$), and PD-1 signaling (36/55 vs 19/173, $P < .001$) (Figure 2*A*). Class comparison between the Immune class and remaining samples identified 112 genes significantly deregulated (Immune Classifier), including 108 overexpressed immune-related genes, such as T-cell receptor components and chemo-attractants for natural killer (NK) and T cells (chemokine [C-C motif] ligand 5 [*CCL5*], chemokine (C-X-C motif) ligand 9 [*CXCL9*], and *CXCL10*, $P < .001$, Supplementary Table 3). Similarly, gene set enrichment analysis identified enrichment of IFN alfa and gamma signaling, inflammatory response (eg, lymphocyte activation, T helper 1-cytotoxic