Genomic Biomarkers to Predict Response to Atezolizumab Plus Bevacizumab Immunotherapy in Hepatocellular Carcinoma: Insights from the IMbrave150 Trial

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Key Words: hepatocellular carcinoma; atezolizumab; bevacizumab; biomarkers, IMbrave150
Abstract

Introduction: Combination immunotherapy, exemplified by atezolizumab plus bevacizumab, has become the standard of care for inoperable hepatocellular carcinoma (HCC). However, the lack of predictive biomarkers and limited understanding of response mechanisms remain a challenge.

Methods: Using data from the IMbrave150plus cohort, we applied an immune signature score (ISS) predictor to stratify HCC patients treated with atezolizumab plus bevacizumab or with sorafenib alone into potential high and low response groups. By applying multiple statistical approaches including a Bayesian covariate prediction algorithm, we refined the signature to 10 key genes (ISS10) for clinical use while maintaining similar predictive power to the full model. We further validated ISS10 in an independent HCC cohort treated with nivolumab plus ipilimumab.

Results: The study identified a significant association between the ISS and treatment response. Among patients classified as high responders, those treated with the atezolizumab plus bevacizumab combination exhibited improved overall and progression-free survival as well as better objective response rate compared to those treated with sorafenib. We also observed a significant correlation between ISS10 and response to nivolumab plus ipilimumab treatment. Analysis of immune cell subpopulations revealed distinct characteristics associated with ISS subtypes. In particular, the ISS10 high subtype displayed a more favorable immune environment with higher proportions of anti-tumor macrophages and activated T-cells, potentially explaining its better response.

Conclusions: Our study suggests that ISS and ISS10 are promising predictive biomarkers for enhanced therapeutic outcomes in HCC patients undergoing combination immunotherapy. These markers are crucial for refining patient stratification and personalized treatment approaches to advance the effectiveness of standard-of-care regimens.

Key words: hepatocellular carcinoma, immunotherapy, atezolizumab, bevacizumab, nivolumab, ipilimumab, genomic predictor.
**Highlight**

- The immune signature score (ISS) is significantly associated with the therapeutic benefit of atezolizumab plus bevacizumab combination therapy in hepatocellular carcinoma (HCC).
- A smaller, 10-gene version of the ISS (ISS10) model maintains strong predictive power, making it a promising tool for clinical application.
- ISS10 shows consistent predictive power across immunotherapy regimens, notably demonstrating a strong association with treatment benefits in HCC patients receiving nivolumab plus ipilimumab combination therapy.
- HCC tumors classified as ISS10 high display an enhanced immune response profile and show a significant association with viral etiology, particularly hepatitis C virus infection.

![Diagram of training and test sets with ISS10 high and low classifications](image)

**ISS10 high**
- Macrophage M1
- Activated CD4 T cells
- CD8 T cells

**ISS10 low**
- Macrophage M2
- NK cells resting
- Mast cells resting

![Graphs showing OS (months) with ISS10 high and low](image)
**Introduction**

Hepatocellular carcinoma (HCC), the most common type of primary liver cancer, represents a major global health burden as both the fastest rising and second deadliest cancer worldwide.\(^1\)\(^-\)\(^4\) Unfortunately, most patients present with intermediate or advanced stage disease, for which few effective therapies exist.\(^1\),\(^5\) However, the recent advent of immunotherapy represents a promising avenue for revolutionizing HCC treatment.\(^6\)\(^-\)\(^8\) Immunotherapy capitalizes on the body’s immune system to recognize and eliminate cancer cells, offering a targeted and potentially more effective approach.\(^6\)

The immune microenvironment in HCC is complex and dynamic, shaped by intricate interactions between cancer cells and the surrounding immune cells.\(^9\) This complexity has fueled the exploration of immunotherapeutic strategies, particularly the advent of immune checkpoint inhibitors (ICIs), which have revolutionized cancer treatment over the past decade.\(^6\) By blocking intrinsic immune-inhibitory ligands or receptors such as programmed death ligand 1 (PD-L1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4), ICIs release the “brakes” on anti-tumor immunity.\(^10\),\(^11\) While ICIs as single agents initially showed marginal benefit in HCC,\(^12\)\(^-\)\(^16\) the combination of the PD-L1 inhibitor atezolizumab with the anti-angiogenic agent bevacizumab has emerged as a breakthrough treatment regimen, showing unprecedented efficacy in the IMbrave150 trial.\(^17\),\(^18\) Based on positive phase III data, this combined immunotherapy gained approval from the US Food and Drug Administration in 2020 as the new first-line standard of care for advanced HCC.

Despite representing a major step forward, the atezolizumab-bevacizumab regimen continues to face significant challenges. Objective response rates remain around 30%, robust predictive biomarkers are lacking, immune-related adverse events can be severe, and prohibitive costs hinder access.\(^16\),\(^18\),\(^19\) Unraveling the complex interplay between the immune system and liver cancer cells is crucial for identifying predictive biomarkers that can distinguish patients who are likely to derive substantial benefit from those who may not respond as favorably. A comprehensive understanding of the genomic landscape holds the key to unlocking the potential of precision medicine in HCC treatment. While the efficacy of atezolizumab plus bevacizumab is evident, identifying biomarkers capable of predicting response can further refine patient selection, facilitate personalized treatment strategies, optimize therapeutic outcomes, and potentially circumvent unnecessary adverse effects.\(^20\)

In the current study, we applied an immune signature score (ISS) prediction model, which was derived from our previous pan-cancer analysis of The Cancer Genome Atlas (TCGA) genomic data,\(^21\) to gene expression data from the IMbrave150 cohort\(^16\),\(^18\),\(^19\) to evaluate the clinical
utility of ISS in the context of atezolizumab plus bevacizumab combination immunotherapy. Identifying biomarkers that enrich for responders to such combination regimens would progress precision oncology in HCC. Our approach to analyzing transcriptomic data provides insight around integral immune processes governing immunotherapeutic susceptibility. The findings could ultimately help spare non-responding patients from ineffective treatment and toxicity and guide more judicious, biomarker-directed allocation of emerging immunotherapies for this prevalent, often fatal malignancy.
Materials and methods

Patients and related RNA expression data

The details of IMbrave150 and GO30140 have been published earlier.\textsuperscript{17, 18, 22} IMbrave150 (NCT03434379) was a phase III clinical trial assessing the safety and efficacy of atezolizumab combined with bevacizumab versus that of sorafenib as the primary treatment for unresectable HCC.\textsuperscript{17, 18} GO30140 (NCT02715531) evaluated the safety and dosage of atezolizumab, both alone and combined with bevacizumab, in patients with HCC.\textsuperscript{22} These studies' primary endpoints were confirmed objective response rate in all patients who received the treatments, and the other key endpoints were overall survival (OS) and progression-free survival (PFS) in the intention-to-treat population, as evaluated using an impulse residue function according to RECIST 1.1.\textsuperscript{23}

Combining the two studies' patient populations yielded 253 patients in the atezolizumab plus bevacizumab treatment arm and 58 patients in the sorafenib treatment arm who had available gene expression data. Those without clinical outcome data were excluded, resulting in the inclusion of 247 patients in the atezolizumab plus bevacizumab arm and 48 patients in the sorafenib arm for the analysis (Figure 1). Since the majority of the data set originates from the IMbrave150 cohort, we will subsequently denote the combined data set as "IMbrave150plus."

RNA sequencing was conducted using Illumina TruSeq RNA Access and analyzed following established procedures as described in a previous study.\textsuperscript{19} The reads from RNA-seq underwent alignment to ribosomal RNA to eliminate ribosomal reads. Subsequently, the remaining reads were aligned to the human reference genome (NCBI Build 38) using GSNAP (v.2013-11-01). Gene expression levels were assessed by counting reads mapped to RefSeq gene exons employing GenomicAlignments. For each cohort, raw gene counts underwent TMM normalization and voom transformation to generate normalized log\textsubscript{2} (CPM) data. Gene features were filtered based on expression and variance thresholds. Gene expression data from IMbrave150 and GO30140 are available in the European Genome-Phenome Archive under the accession number EGAD00001008128.

We also analyzed an independent HCC cohort treated with a different immunotherapy combination. Gene expression data and clinical data from a Taipei HCC cohort were obtained from the NCBI Gene Expression Omnibus site under accession number GSE140901.\textsuperscript{25} The Taipei cohort predominantly consisted of HCC patients treated with either nivolumab monotherapy (n = 13) or a combination of nivolumab and ipilimumab (n = 9). From the initial 24 patients, we excluded 2 who received a markedly different treatment regimen (spartalizumab and sabatolimab combination), ensuring a more homogeneous treatment group for our analysis. Finally, gene...
expression data and clinical data including viral etiology from TCGA HCC cohort were obtained from cBioPortal (https://www.cbioportal.org/).  

**Immune signature**

We extracted a previously identified set of 105 genes used in the ISS prediction model from RNA-seq data of HCC tumors in the TCGA project (TCGA-LIHC). This gene set from LIHC data served as the training set during the development of prediction models. Subsequently, the signature was employed to stratify patients in IMbrave150plus. During the development of prediction models using the IMbrave150plus data set, 8 genes from the original training set were excluded due to variations in RNA-seq data processing. In essence, the expression data for the remaining 97 ISS genes in the training set were combined to construct a classifier using 6 different algorithms to stratify the patients into ISS high or low subtypes.

**Data analysis**

The mRNA expression data that were collected were normalized and transformed in line with previous studies. BRB-ArrayTools (v 4.6.2), a freeware program from the National Institutes of Health (https://brb.nci.nih.gov/BRB-ArrayTools/), was used to construct predictive models to evaluate the clinical relevance of ISS. Cluster (v 3.0) and TreeView (v 1.6) were employed to generate a heatmap of gene expression data. The R language (http://www.r-project.org, v 4.1.1) was used for statistical analysis.

Prior to pooling RNA expression data from TCGA-LIHC and IMbrave150plus for predictive purposes, expression data from both data sets were independently converted to z-scores ($z=(x - \text{mean})/\text{standard deviation}$). Six independent prediction algorithms were used to categorize individual human HCC samples into ISS high or ISS low subtypes. These algorithms included linear discriminant analysis, support vector machines, nearest centroid, nearest neighbor, compound covariate predictor, and Bayesian compound covariate predictor (BCCP). In the BCCP prediction, the classifier estimated the probability that an individual patient possessed either a high or low immune signature, utilizing a Bayesian probability of IS score cutoff at 0.5. This cutoff was optimized by comparing response rates to immunotherapies in previous studies.

**Identification of minimum number of genes for prediction model**

To determine the minimum number of genes required for our prediction model without compromising its accuracy, we employed two approaches to evaluate the impact of using fewer genes during training and on predicted clinical outcomes. First, we evaluated the miscalculation
rate during prediction using a reduced number of genes in the training set. Utilizing the pamr algorithm,\textsuperscript{38} we assessed the miscalculation rate across different gene counts and observed a significant increase in miscalculation rate when the predictor used fewer than 10 genes. This analysis suggested that 10 genes offered a good balance between model complexity and accuracy.

In the second approach, we employed a stepwise iterative methodology to assess the impact of removing genes from the prediction models on the predicted outcomes in the IMbrave150plus data set. Initially, we ranked the 97 genes in the ISS signature based on their expression ratio between the high and low subtypes using the TCGA training data. We began with a 97-gene BCCP model and systematically eliminated the bottom 10 genes from the rank list in each iteration. After removing each set of 10 genes, we refit Cox proportional hazards models and calculated hazard ratios (HRs) for OS prediction. This process enabled us to evaluate the prediction performance across iterative models containing 90, 80, 70 genes, and so on, until we reached a final model with only the top 10 genes. The HRs were used to assess the significance of each gene removal step on survival prediction. By gauging the HRs, we were able to determine the impact of removing specific genes on OS prediction, allowing us to find minimum gene set for accurate prediction.

**Analysis of immune cell population using CIBERTSORT**

To unravel the composition of immune cell populations within each HCC tumor, we employed CIBERTSORT,\textsuperscript{39} a computational method widely used for analyzing immune cell composition in cancer cells. The deconvolution algorithm of CIBERTSORT uses bulk gene expression data to estimate the relative proportions of different immune cell types in a mixed cell population. It relies on a reference gene signature matrix comprising gene expression profiles of 22 functionally distinct immune cell types. By comparing the gene expression profile of a mixed cell population to the reference matrix, CIBERTSORT can estimate the relative proportions of each immune cell type present in the sample.
Results

ISS is significantly associated with therapeutic benefit of combination treatment with atezolizumab and bevacizumab

Using an immune signature derived from prior analyses involving pan-cancer-wide examination of genomic data from the TCGA project, we formulated several prediction models. These models were designed to assess the consistency and resilience of predictions, aiming to categorize HCC tumors within the IMbrave150plus cohort into potential groups with high and low response to the combination therapy of atezolizumab and bevacizumab (Figure 2A).

Kaplan-Meier plots and log-rank tests on OS data revealed that patients identified as high responders by the BCCP model exhibited better OS and PFS outcomes with atezolizumab and bevacizumab treatment compared to sorafenib (Figure 2B, C). This trend persisted across all 6 prediction models (Supplementary Figure 1). In contrast, patients predicted as low responders showed no significant difference in OS or PFS between the 2 treatment arms. These results demonstrate the ability of gene expression–based predictors to identify patients likely to respond to the combination treatment with atezolizumab and bevacizumab, underscoring the strong association between the ISS and treatment response.

Consistently, Cox proportional hazard regression analysis also revealed a significant advantage of the combination treatment over sorafenib, specifically among patients classified under the ISS high subtype. This advantage was seen in OS among patients classified under the ISS high subtype (HR 0.263, 95% CI 0.135–0.51, \( P=7.78 \times 10^{-5} \)) but not those classified under the ISS low subtype (HR 0.875 95% CI 0.46–1.66, \( P=0.683 \); Figure 3). Similar findings were observed in PFS (ISS high subtype, HR 0.496, 95% CI 0.275–0.894, \( P=0.019 \); ISS low subtype, HR 0.948, 95% CI 0.599–1.499, \( P=0.82 \); Figure 3).

Overall, these results demonstrate that the combination treatment significantly prolongs OS and PFS compared to sorafenib monotherapy specifically in patients with the ISS high subtype and further suggest that the benefit of the combination treatment might be attributable to differences in the underlying tumor immune microenvironment between subtypes.

Development of ISS10

To accelerate the clinical application of our findings using simpler technology platforms such as real-time PCR or NanoString, we aimed to streamline our prediction models by reducing the number of genes without compromising predictive power. Our goal was to identify the smallest gene signature that maintains strong prediction of clinical outcomes, as smaller gene panels lower the cost and increase the accessibility of data generation on routine clinical platforms.
Our assessment of miscalculation rates during training identified 10 genes as the optimal number for constructing the predictor (Supplementary Figure 2A). Next, our stepwise iterative gene reduction process yielded the ISS10 gene signature (Figure 4A). Patients in the ISS10 high subtype maintained an OS advantage with the combination treatment versus sorafenib (HR 0.2805, 95% CI 0.145–0.54, \( P=1.4 \times 10^{-4} \)), consistent with the full 97-gene model (HR 0.2628, 95% CI 0.135–0.51, \( P=7.78 \times 10^{-5} \); Supplementary Figure 2B). Conversely, models with more than 10 genes did not yield superior outcomes in predicting responders to the combination treatment.

Subsequent analysis with predicted outcomes of ISS10 showed non-inferior outcomes to the prediction with the full gene set. Kaplan-Meier plots and log-rank tests on OS data revealed that patients identified as high responders by the ISS10 prediction model exhibited enhanced OS with atezolizumab and bevacizumab treatment compared to sorafenib (Figure 4B). In contrast, patients predicted as low responders showed no significant difference in overall OS between the two treatment arms. Likewise, we observed a similar significant difference in PFS between predicted high and low responders (Figure 4B). In contrast with the ISS10 high subtype, there was no discernible OS benefit observed from the combination treatment among patients classified under the ISS10 low subtype (HR 0.883, 95% CI 0.46–1.71, \( P=0.712 \); Figure 4C). Similar findings were observed in PFS (ISS10 high subtype, HR 0.5045, 95% CI 0.2875–0.8854, \( P=0.017 \); ISS10 low subtype, HR 0.938, 95% CI 0.5844–1.508, \( P=0.79 \)).

In alignment with these findings, when patients were categorized into ISS10 high and low subtypes in the context of responders and non-responders, those in the high subtype group showed significantly better responses only when treated with the combination of atezolizumab and bevacizumab (\( P=8.6 \times 10^{-5} \) by \( \chi^2 \)-test in Table 1 and \( P=5.0 \times 10^{-4} \) by \( \chi^2 \)-test in Supplementary Table 1). These results underscore the potential of the ISS10 signature as a predictive biomarker for treatment response, particularly in patients receiving atezolizumab and bevacizumab combination therapy. The ISS10 signature effectively stratifies patients into high and low responders, demonstrating its utility in identifying individuals likely to benefit from this treatment regimen. Taken together, these results demonstrate that the ISS10 model sufficiently identifies combination treatment responders without sacrificing accuracy compared to the full gene signature.

**Significance of ISS10 in HCC cohort treated with nivolumab and ipilimumab**

To validate the predictive capacity of ISS10 in HCC patients treated with different ICIs, we analyzed gene expression data from the Taipei cohort. This cohort comprised HCC patients
treated with either nivolumab monotherapy (n=13) or a combination of nivolumab and ipilimumab (n=9). Our analysis revealed that patients classified with the ISS10 high subtype exhibited significantly prolonged OS and a higher proportion with partial response or stable disease (Figure 5A and 5C, Supplementary Table 2). Notably, the predictive power of ISS10 was particularly pronounced in patients receiving the combination therapy, as demonstrated by more distinct survival curve separation (Figure 5B) and an increased fraction of patients responding to the treatment (Supplementary Table 2). These findings further validate ISS10’s robust predictive capability across different ICI regimens in HCC patients, potentially offering valuable guidance for clinical decision-making.

Immune cell subpopulations in ISS10 subtypes

To gain insights into the biological basis for the better response of the ISS10 high subtype of HCC tumors to the combination therapy, we conducted an in-depth analysis of the immune cell subpopulations present in these tumors. Specifically, we used the CIBERSORT algorithm to estimate the percentage of immune cells in these tumors by analyzing their gene expression data (Figure 6). Our analysis revealed that the ISS10 high subtype had a significantly higher fraction of anti-tumor macrophage M1, while the ISS10 low subtype had a significantly higher proportion of pro-tumor macrophage M2 and undifferentiated macrophage M0 (Supplementary Figure 3). These findings suggest that the absence of active anti-cancer macrophages may contribute to the poor response of ISS10 low tumors to the combination therapy. Furthermore, the ISS10 low subtype was characterized by a higher fraction of resting mast cells and natural killer (NK) cells, indicating a more immunologically quiescent and less inflamed tumor microenvironment. This quiescent state may lead to less active anti-tumor immunity in this subtype, leading to the poor response to checkpoint inhibition. In contrast, the ISS10 high subtype was characterized by a higher fraction of CD8 T-cells, CD4 memory activated T-cells, and follicular helper T-cells. These findings indicate that the immune microenvironment in the ISS10 high subtype is primed to recognize and eliminate tumor cells. Therefore, ICI treatment may further stimulate anti-tumor immunity, leading to a more robust response to the combination therapy. Overall, our analysis suggests that the differential distribution of immune cell subpopulations in the ISS10 subtypes may underlie their distinct responses to the combination therapy. These findings highlight the importance of understanding the tumor microenvironment and the immune landscape in guiding the development of effective immunotherapeutic strategies for HCC.

Biological and etiological characteristics in ISS10 high subtypes
To gain in-depth understanding of the biological processes at play in the ISS10 high subgroup, we employed Ingenuity Pathway Analysis to identify genes that were differentially expressed between ISS10 high and low HCC tumors in both the TCGA and IMbrave150plus cohorts (Figure 7A). As expected, the analysis revealed a strong emphasis on immune response pathways in the ISS10 high subtype. These pathways included NK cell signaling, communication between dendritic cells and NK cells, interactions between immune and non-immune cells, interferon gamma signaling, T helper cell differentiation (Th1/Th2), and signaling pathways mediated by interleukin-27, interleukin-10, and macrophage activation (Figure 7B). These findings align with the notion that the ISS10 high subtype exhibits a heightened immune response, potentially explaining its improved response to the combination therapy. Further exploration of these pathways has the potential to unveil additional insights into HCC biology and guide the development of novel and targeted immunotherapies.

Finally, given that hepatitis B virus (HBV) and hepatitis C virus (HCV) are prevalent causes of HCC in various geographical regions,\textsuperscript{44, 45} we investigated the potential relationship between viral etiology and ISS10 subtype using gene expression and clinical data from the TCGA study.\textsuperscript{24} Our analysis revealed a notable association between viral etiology and the ISS10 high subtype (Supplementary Figure 4A, Supplementary Table 3). This association was particularly pronounced in patients with HCV infection and even more striking in cases of dual infection with both HBV and HCV. These findings suggest that HCC patients with viral etiologies, especially those related to HCV or dual HBV and HCV infections, may derive greater benefit from ICI therapy compared to patients with non-viral HCC etiologies. To further validate this observation, we examined the relationship between viral etiology and ISS10 subtype in the Taipei cohort. Consistent with our findings in the TCGA cohort, we observed a trend where a larger proportion of HCC patients with HCV infection were classified into the ISS10 high subtype (Supplementary Figure 4B and Supplementary Table 4). This corroborating evidence strengthens the potential link between viral etiology, particularly HCV, and the ISS10 high subtype, which may have implications for predicting responsiveness to ICI therapy in HCC patients.
Discussion
In this study, we explored the clinical utility of ISS derived from TCGA pan-cancer genomic analyses within the context of atezolizumab plus bevacizumab combination therapy in HCC. Our analysis, based on gene expression data from the IMbrave150plus cohort, aimed to identify predictive biomarkers that could refine patient selection and optimize therapeutic outcomes. Our findings demonstrate a significant association between ISS and the therapeutic benefit of atezolizumab plus bevacizumab combination treatment. Multiple prediction models consistently identified an ISS high subgroup of HCC patients exhibiting improved OS and PFS on atezolizumab and bevacizumab compared to sorafenib. In contrast, ISS low HCCs failed to show survival advantage with the immunotherapy combination over sorafenib. These findings highlight the integral role of the tumor immune microenvironment in determining therapeutic outcomes and the potential of the ISS to prospectively identify patients likely to benefit from this emerging first-line regimen.

To expedite clinical application, the original 97-gene signature was refined and streamlined down to 10 key genes (ISS10). Rigorous stepwise gene reduction and misclassification analyses verified that 10 was the minimum number of genes required to maintain accurate predictive power. The ISS10 model could effectively identify responders to the immunotherapy combination without sacrificing precision compared to the larger signature. This more focused 10-gene biomarker panel offers a practical and accessible clinical tool to guide more precise application of atezolizumab and bevacizumab versus sorafenib based on the tumor immune profile. Therefore, the ISS10 model will lower cost barriers and technical complexity, providing a more feasible approach for clinical implementation compared to the full multi-gene signature.

Importantly, the predictive power of ISS10 extends beyond the atezolizumab and bevacizumab combination therapy. Among HCC patients treated with nivolumab alone or in combination with ipilimumab, those classified with the ISS10 high subtype demonstrated significantly longer survival and better response to their therapy. Notably, this association was more pronounced in patients receiving the nivolumab and ipilimumab combination. This finding is particularly significant given that this combination was approved for second-line HCC treatment following the CheckMate 040 trials, and recent interim analysis of the CheckMate 9DW phase III trial indicated the combination’s superiority to lenvatinib or sorafenib in first-line treatment. These results suggest that ISS10 could serve as a valuable tool for guiding ICI treatment selection in HCC patients across different immunotherapy regimens.
Our examination of immune cell subpopulations unveiled contrasting profiles between ISS10 high and low subtypes. The ISS10 high subtype exhibited enrichment in anti-tumor M1 macrophages and activated T-cell subsets, implying a microenvironment conducive to effective immunity in baseline tumor tissues. Conversely, the ISS10 low subtype demonstrated an abundance of immunosuppressive M2 macrophages and quiescent NK cells, indicating immune quiescence and potentially explaining their limited response to the combination therapy. In-depth pathway analysis of genes correlated with two subtypes further elucidated the immune-related pathways enriched in ISS10 high tumors, providing insights into the molecular mechanisms underlying treatment response. These pathways, including NK cell signaling and interferon gamma signaling, underscore the importance of immune activation in mediating therapeutic efficacy. Moreover, our analysis into the biological characteristics of ISS10 subtypes uncovered associations with HS cell traits, highlighting the interplay between immune activity and tumor biology. ISS10 high tumors exhibited elevated HS cell features, suggesting that genetic traits of HS cells may trigger the activation of immune cells in the tumor microenvironment. These findings shed light on the underlying biology of HCC and offer insights into potential mechanisms driving treatment response.

Our investigation into the relationship between viral etiology and the ISS10 subtype in HCC revealed intriguing results. We found a significant correlation between viral etiology, particularly HCV infection, and the ISS10 high subtype. This association was even more pronounced in cases of co-infection with both HCV and HBV. These findings suggest that HCC patients with viral backgrounds, especially those linked to HCV or dual HBV/HCV infections, might be more responsive to ICI therapy. Our observations are consistent with previous studies that demonstrated improved survival rates in HBV- or HCV-related HCC patients receiving ICI treatment compared to those with non-viral HCC.48, 49 However, the literature on this topic presents some inconsistencies. While our findings and some earlier studies suggest a potential advantage for patients with viral HCC in ICI treatment outcomes, more recent meta-analyses did not find a clear connection. A comprehensive review of randomized clinical trials of ICIs in advanced HCC, along with a separate analysis of multiple ICI trials, failed to establish a clear link between viral etiology and ICI treatment efficacy.50-52 These conflicting results underscore the complexity of the relationship between HCC etiology and immunotherapy outcomes, suggesting the presence of additional, as-yet-unidentified factors influencing treatment response.

While our ISS originated independently from the IMbrave150 study, derived from the analysis of the TCGA data set in a pan-cancer manner, the performance of the ISS in identifying potential responders to combination therapy in the IMbrave150plus cohort was highly comparable
to that of the atezolizumab plus bevacizumab response signature (ABRS).<sup>19</sup> Notably, ABRS genes were identified through differential expression analysis between responders and non-responders in the GO30140 and IMbrave150 trial. Because the genes in ABRS were identified through differential expression analysis between responders and non-responders in the GO30140 and IMbrave150 trial, it is expected that patients with high expression of ABRS genes would have longer PFS (HR 0.49, 95% CI 0.25–0.97, \(P=0.041\)) and OS (HR 0.26, 95% CI 0.11–0.58, \(P=0.0012\)) than those with low expression. Interestingly, our ISS10 achieved similar efficacy in predicting response, showing non-inferior outcomes to those of ABRS with pre-selected responder genes (PFS, HR 0.5045, 95% CI 0.2875–0.8854, \(P=0.017\); OS, HR 0.2805, 95% CI 0.145–0.54, \(P=1.4 \times 10^{-4}\)), suggesting its ability to effectively capture underlying immunological features in the tumor microenvironment. Interestingly, despite similar efficacy, none of the genes in ISS10 (ITGAL, IKZF3, CD8A, TOX, GBP5, PRF1, CCL5, LCK, SLAMF7, and CXCL9) overlap with those in ABRS (CXCR2P1, ICOS, TIMD4, CTLA4, PAX5, KLRC3, FCRL3, AIM2, GBP5, and CCL4).<sup>19</sup> This suggests that ISS10 captures distinct, yet potentially complementary, aspects of the tumor immune microenvironment, contributing to its comparable effectiveness in predicting response to the combination therapy with atezolizumab and bevacizumab and to the combination with nivolumab and ipilimumab.

This study has several limitations that should be considered when interpreting the results. First, the findings require prospective validation in additional independent cohorts to firmly establish the clinical utility of the 10-gene signature. The study was conducted retrospectively on data from previously completed clinical trials. Validating the signature’s predictive power prospectively is essential before routine clinical use. Second, the study population included only advanced HCC patients eligible for first-line systemic therapy. Whether the signature predicts immunotherapy benefit in patients with earlier-stage disease merits further investigation. Third, the current study used transcriptomic data alone for prediction. Incorporating complementary biomarkers from immunohistochemical analysis to measure protein expression could potentially enhance accuracy. Fourth, the association of ISS10 with the clinical outcome of the combination therapy with nivolumab and ipilimumab requires further validation using data from a randomized clinical trial like CheckMate 9DW, as the number of patients in the Taipei cohort is very small and do not represent a randomized trial. Overall, further clinical validation cohorts, experimental functional studies, and assay standardization will maximize clinical impact moving forward. However, the current study provides proof-of-concept for an immunogenomic biomarker predicting immunotherapy outcomes in HCC patients.
In summary, this research illustrates that the ISS can function as a predictive biomarker for identifying HCC patients who are likely to experience improved survival outcomes in response to atezolizumab and bevacizumab combination therapy compared to sorafenib and in response to nivolumab and ipilimumab combination therapy. By narrowing down the signature to 10 key genes, a concise panel has been established that maintains accuracy in guiding treatment decisions and is easily applicable in clinical settings. This biomarker also revealed valuable biological insights by highlighting distinct immune cell and etiology characteristics linked to the response to combination therapy. In future studies, it will be crucial to validate the ISS10 panel prospectively in additional patient groups and to conduct head-to-head comparisons with other proposed signatures to firmly establish its usefulness. Overall, the clinical translation of biomarkers that identify immunotherapy responders remains essential for enhancing survival rates in advanced HCC by precisely matching patients with treatments based on their biology. Subsequent investigations can expand upon these findings by refining and prospectively validating the predictive model while exploring actionable mechanisms associated with primary immunotherapy resistance.

Acknowledgements
This work is supported by the NIH/NCI under award numbers R01CA237327, P50CA217674, and P30CA016672; the Duncan Family Institute for Cancer Prevention and Risk Assessment Seed Funding Research Program at MD Anderson (2016 cycle); institutional bridge funds from MD Anderson (2022 cycle); an Institutional Research Grant from MD Anderson (2021 cycle); The Research Supporting Program of The Korean Association for the Study of the Liver and The Korean Liver Foundation; a Korea University Hospital Research Grant and Industrial Strategic Technology Development Program (20024893, Development of non-thermal dynamic focusing focused ultrasound therapy device with integrated ultrasound image Therapy Device) funded By the Ministry of Trade, Industry & Energy, Korea; the National Research Foundation of Korea (NRF) grant funded by the Korea government (Ministry of Science and ICT, 2023R1A2C10073311212982036810102). Editorial support was provided by Bryan Tutt and Sarah J. Bronson from the Research Medical Library at UT MD Anderson Cancer Center.
FOOTNOTES

Authors’ contribution

Conceptualization: SYY, SHL, JHK, JSL.
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Data curation: SWB, BS, YSJ, SHK, KP, ISC, HP.
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Visualization: SHL, HP, KP, JSL.
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Funding acquisition: JSL, JHK, SYY, SHL
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All the authors approved this version of the manuscript to be published.

Conflicts of Interest

The authors have no conflicts to disclose.
References


**Abbreviations**

HCC, hepatocellular carcinoma  
ICI, immune checkpoint inhibitor  
ISS, immune signature score  
TCGA, The Cancer Genome Atlas  
OS, overall survival  
PFS, progression free survival  
BCCP, Bayesian compound covariate predictor  
HR, hazard ratio  
CI, confidence interval
Table 1. Contingency table depicting the association of ISS10 signature with atezolizumab and bevacizumab or sorafenib treatment in IMbrave150plus cohort.

<table>
<thead>
<tr>
<th>ISS10 Prediction</th>
<th>Responder (CR/PR)</th>
<th>Non-Responder (SD/PD)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Atezolizumab and bevacizumab treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>high</td>
<td>53 (65.4%)</td>
<td>63 (38%)</td>
<td>116 (47%)</td>
</tr>
<tr>
<td>low</td>
<td>28 (34.6%)</td>
<td>103 (62%)</td>
<td>131 (53%)</td>
</tr>
<tr>
<td>Total</td>
<td>81 (100%)</td>
<td>166 (100%)</td>
<td>247 (100%)</td>
</tr>
</tbody>
</table>

\[ P = 8.6 \times 10^{-5} \text{ by } \chi^2\text{-test} \]

| **Sorafenib treatment** |                   |                      |       |
| high                     | 6 (60%)           | 18 (47.4%)           | 24 (50%) |
| low                      | 4 (40%)           | 20 (52.6%)           | 24 (50%) |
| Total                    | 10 (100%)         | 38 (100%)            | 48 (100%) |

\[ P = 0.72 \text{ by } \chi^2\text{-test} \]

CR, complete response
PR, partial response
SD, stable disease
PD, progressed
Figure 1. Selection process for IMbrave150plus (IMbrave150 and GO30140) cohort.
Ate, atezolizumab; Bev, bevacizumab; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.
Figure 2. Enhanced efficacy of combination therapy in ISS high subtype.
(A) This figure illustrates the use of predictive models to stratify patients with HCC in a IMbrave150plus cohort. The models are used to identify patients who are likely to benefit from combination immunotherapy with atezolizumab and bevacizumab.
(B,C) Kaplan-Meier plots of overall survival (B) and progression-free survival (C) show that patients in the ISS high subtype have significantly better overall and progression-free survival when treated with the combination therapy compared to sorafenib.
LOOCV, leave one out cross validation; CCP, compound covariate predictor; LDA, linear discriminant analysis; NN, nearest neighbor; NC, nearest centroid; SVM, support vector machines; BCCP, Bayesian compound covariate predictor; Ate, atezolizumab; Bev, bevacizumab; ISS, immune signature score; OS, overall survival, PFS, progression-free survival.
A

TCGA Training Set

Development and training of classifiers

(CCp, LDA, 1NN, 3NN, NC, SVM, BCCP)

Imbrave150plus

Ate + Bev, n = 247
Sorafenib, n = 48

Prediction

B

ISS high

Ate + Bev (n = 114)
Sorafenib (n = 20)

P = 2.0 x 10^-5

OS (months)

C

ISS high

Ate + Bev (n = 114)
Sorafenib (n = 20)

P = 0.02

PFS (months)

ISS low

Ate + Bev (n = 114)
Sorafenib (n = 28)

P = 0.7

OS (months)

ISS low

Ate + Bev (n = 133)
Sorafenib (n = 28)

P = 0.8

PFS (months)
Figure 3. Enhanced efficacy of combination therapy in ISS high subtype.

Forest plots display the hazard ratios (HRs) for overall survival and progression-free survival of patients with hepatocellular carcinoma treated with the atezolizumab-bevacizumab combination compared to sorafenib, based on their ISS subtype. The plots show that patients in the ISS high subtype have a significantly lower HR for overall survival and progression-free survival when treated with the combination therapy compared to sorafenib. The dotted lines represent the 95% confidence intervals (CIs) of HRs. The study used a Cox proportional hazard regression model to analyze the interaction between overall survival and progression-free survival of patients with ISS high and low subtypes and the combination treatment. ISS, immune signature score; Ate, atezolizumab; Bev, bevacizumab; OS, overall survival; PFS, progression-free survival.
Figure 4. Predictive performance of ISS10.

(A) The matrix displays the expression patterns of the 10 genes within the ISS10 signature in the TCGA training and IMbrave150plus prediction set. Each cell in the matrix represents the expression level of a gene feature in an individual tissue. The red and green colors in the cells indicate relative high and low expression levels, respectively.

(B) Kaplan-Meier plots compare the overall survival and progression-free survival of patients treated with the combination therapy or sorafenib, based on their ISS10 subtype. The plots show that patients in the ISS10 high subtype have significantly better overall and progression-free survival when treated with the combination therapy compared to sorafenib.

(C) Forest plots show the hazard ratio (HRs) for overall survival and progression-free survival of patients with ISS10 high and low subtypes treated with the atezolizumab-bevacizumab combination compared to sorafenib. The dotted lines represent the 95% confidence intervals (CIs) of HRs. The study used a Cox proportional hazard regression model to analyze the interaction between overall survival and progression-free survival of patients with ISS10 high and low subtypes and the combination treatment.

ISS10, 10-gene immune signature score; OS, overall survival; PFS, progression free survival. Ate, atezolizumab; Bev, bevacizumab; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.
A  
Training  
ISS10 high  
ISS10 low  
Treatment  
Ate+Bev  
Sor  

Prediction  

B  
ISS10 high  
Ate + Bev  
Sorafenib  
(n = 116)  
(n = 24)  
P = 5.0 x 10^{-4}  

ISS10 low  
(n = 131)  
(n = 24)  
P = 0.7  

C  
OS  
high  
low  


PFS  
high  
low  

Hazard Ratio for PFS or OS, 95% CI (log10)
Figure 5. Significance of ISS10 in HCC patients treated with nivolumab and ipilimumab.

(A) Kaplan-Meier plots of overall survival show that patients in the ISS10 high subtype have significantly better overall survival than those in the ISS10 low subtype when treated with either nivolumab monotherapy (n=13) or the combination of nivolumab and ipilimumab (n=9).

(B) Kaplan-Meier plots of overall survival show that patients in the ISS10 high subtype have significantly better overall survival than those in the ISS10 low subtype when treated with the combination of nivolumab and ipilimumab (n=9). The predictive power of ISS10 was greater in the combination treatment.

(C) Stacked bar plots of the percentages of the patients in ISS10 subtypes for the 3 response categories. OS, overall survival; PR, partial response; SD, stable disease; PD, progressed. P-value is estimated by chi-square test.
Figure 6. Immune characteristics of hepatocellular carcinoma tumors in ISS10 subtypes.
(A) The stacked bar plots display the infiltrated immune cells estimated by the CIBERSORT algorithm in the pooled cohort treated with the combination of atezolizumab and bevacizumab. The plots show that the ISS10 high subtype has a higher fraction of macrophage M1 subset compared to the ISS10 low subtype. All samples were sorted by macrophage M1 fraction.
(B) The volcano plot represents enriched immune cells in the ISS10 high subtype over the ISS10 low subtype. The plot shows that several immune cells are enriched in the ISS10 high subtype, including T-cells, macrophages, and dendritic cells. The red dotted line on the y-axis indicates a P-value of 0.05. The study used the Wilcoxon rank-sum test to calculate P-values and adjusted for multiple testing.
Figure 7. Enriched signaling pathways in ISS10 high subtype. (A) A Venn diagram displays the overlap between gene lists from two independently identified genes associated with ISS10 subtypes in the TCGA and IMbrave150plus cohorts. The diagram shows a significant overlap between the gene lists, indicating that the ISS10 high subtype is characterized by a distinct gene expression profile. (B) The figure shows the top 20 enriched signaling pathways in the ISS10 high subtype, as identified by Ingenuity Pathway Analysis. Fisher’s exact test was applied to gene sets defined in the Ingenuity Pathway Analysis database to identify enriched signaling pathways in the ISS10 high subtype. The results show that various immune-associated pathways are enriched in the ISS10 high subtype, including pathways related to immune cell activation, cytokine signaling, and inflammation.
Supplementary Figure 1. Consistency of ISS subtype across six prediction models. Kaplan-Meier plots display the overall survival and progression-free survival of patients treated with the atezolizumab-bevacizumab combination or sorafenib, stratified by ISS high and low subtypes from 6 independent prediction models. The figure shows that the ISS subtype has persistent significance for survival across six independent prediction models.

CCP, compound covariate predictor; LDA, linear discriminant analysis; 1NN, one nearest neighbor; 3NN, three nearest neighbor; NC, nearest centroid; SVM, support vector machines; Ate, atezolizumab; Bev, bevacizumab; OS, overall survival; PFS, progression-free survival.
Supplementary Figure 2. Evaluation of prediction models with varying numbers of genes.

(A) The figure displays the miscalculation rate of prediction models with a given number of genes. Gene expression data from TCGA were used to estimate the minimum number of genes for the prediction model during leave-one-out cross-validation. The results show that prediction models with 10 genes have an acceptable rate of miscalculation (15%).

(B) The forest plots illustrate the hazard ratios (HRs) for overall survival regarding atezolizumab-bevacizumab combination treatment versus sorafenib treatment in ISS high subtypes, derived from prediction models featuring different numbers of genes in the IMbrave150plus cohort. The dotted lines denote the 95% confidence intervals (CIs) of HRs. Ate, atezolizumab; Bev, bevacizumab; OS, overall survival.
Supplementary Figure 3. Immune cell fractions in hepatocellular carcinoma tumors of ISS10 subtypes from the patients treated with the combination of atezolizumab and bevacizumab. * $P<0.05$. 
Supplementary Figure 4. Association of the ISS10 subtypes with viral etiology in HCC patients.

Stacked bar plots depict the percentages of the patients for the different viral etiology categories predicted by the ISS10 in TCGA cohort (A) and Taipei cohort (B). None, no viral infection; HBV, hepatitis B virus; HCV, hepatitis B virus; HBV + HCV, dual infection of HBV and HCV. P-value is estimated by chi-square test.
**Supplementary Table 1.** Contingency table depicting the significant association of ISS10 predictor with atezolizumab and bevacizumab or sorafenib treatment in IMbrave150plus cohort.

<table>
<thead>
<tr>
<th>Prediction</th>
<th>CR</th>
<th>PR</th>
<th>SD</th>
<th>PD</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Atezolizumab and bevacizumab treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>17</td>
<td>36</td>
<td>42</td>
<td>21</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>(74%)</td>
<td>(62%)</td>
<td>(38.9%)</td>
<td>(36.2%)</td>
<td>(47%)</td>
</tr>
<tr>
<td>Low</td>
<td>6</td>
<td>22</td>
<td>66</td>
<td>37</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>(26%)</td>
<td>(38%)</td>
<td>(61.1%)</td>
<td>(63.8%)</td>
<td>(53%)</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>58</td>
<td>108</td>
<td>58</td>
<td>247</td>
</tr>
<tr>
<td></td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
</tr>
</tbody>
</table>

$P = 5.0 \times 10^{-4}$ by $\chi^2$-test

<table>
<thead>
<tr>
<th>Prediction</th>
<th>CR</th>
<th>PR</th>
<th>SD</th>
<th>PD</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sorafenib treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>0</td>
<td>6</td>
<td>11</td>
<td>7</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>(0%)</td>
<td>(60%)</td>
<td>(45.8%)</td>
<td>(50%)</td>
<td>(50%)</td>
</tr>
<tr>
<td>Low</td>
<td>0</td>
<td>4</td>
<td>13</td>
<td>7</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>(0%)</td>
<td>(40%)</td>
<td>(54.2%)</td>
<td>(50%)</td>
<td>(50%)</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>10</td>
<td>24</td>
<td>14</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>(0%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
</tr>
</tbody>
</table>

$P = 0.753$ by $\chi^2$-test

PR, partial response
SD, stable disease
PD, progressed
Supplementary Table 2. Contingency table depicting the significant association of ISS10 predictor with nivolumab monotherapy or the combination of nivolumab and ipilimumab treatment in Taipei cohort.

<table>
<thead>
<tr>
<th>Prediction</th>
<th>PR</th>
<th>SD</th>
<th>PD</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All Patients (n = 22)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>5 (63.3%)</td>
<td>3 (33.3%)</td>
<td>0 (0%)</td>
<td>8 (36.4%)</td>
</tr>
<tr>
<td>Low</td>
<td>1 (16.6%)</td>
<td>6 (66.7%)</td>
<td>7 (100%)</td>
<td>14 (63.6%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>6 (100%)</td>
<td>9 (100%)</td>
<td>7 (100%)</td>
<td>22 (100%)</td>
</tr>
</tbody>
</table>

$P = 0.007$ by $\chi^2$-test

<table>
<thead>
<tr>
<th></th>
<th>PR</th>
<th>SD</th>
<th>PD</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nivolumab monotherapy (n = 13)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>3 (75%)</td>
<td>2 (40%)</td>
<td>0 (0%)</td>
<td>5 (38.5%)</td>
</tr>
<tr>
<td>Low</td>
<td>1 (25%)</td>
<td>4 (80%)</td>
<td>3 (100%)</td>
<td>8 (61.5%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>4</td>
<td>6 (100%)</td>
<td>3 (100%)</td>
<td>13 (100%)</td>
</tr>
</tbody>
</table>

$P = 0.12$ by $\chi^2$-test

<table>
<thead>
<tr>
<th></th>
<th>PR</th>
<th>SD</th>
<th>PD</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nivolumab and ipilimumab (n = 9)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>2 (100%)</td>
<td>1 (33.3%)</td>
<td>0 (0%)</td>
<td>3 (33.3%)</td>
</tr>
<tr>
<td>Low</td>
<td>0 (0%)</td>
<td>2 (66.7%)</td>
<td>4 (100%)</td>
<td>6 (66.7%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>2 (100%)</td>
<td>3 (100%)</td>
<td>4 (100%)</td>
<td>9 (100%)</td>
</tr>
</tbody>
</table>

$P = 0.049$ by $\chi^2$-test

PR, partial response
SD, stable disease
PD, progressed
**Supplementary Table 3.** Contingency table depicting the association of ISS10 subtypes with viral etiology in TCGA cohort (n = 371).

\[
\begin{array}{|c|c|c|c|c|c|}
\hline
\text{Prediction} & \text{None} & \text{HBV} & \text{HCV} & \text{Both} & \text{Total} \\
\hline
\text{High} & 99 (45.4\%) & 47 (48.5\%) & 30 (61.2\%) & 6 (85.7\%) & 182 (49\%) \\
\text{Low} & 119 (54.6\%) & 50 (51.5\%) & 19 (38.8\%) & 1 (14.3\%) & 189 (51\%) \\
\hline
\text{Total} & 218 (100\%) & 97 (100\%) & 49 (100\%) & 7 (100\%) & 371 (100\%) \\
\hline
\end{array}
\]

\( P = 0.049 \) by \( \chi^2 \)-test
**Supplementary Table 4.** Contingency table depicting the association of ISS10 predictor with viral etiology in Taipei cohort (n = 22).

<table>
<thead>
<tr>
<th>Prediction</th>
<th>None</th>
<th>HBV</th>
<th>HCV</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>1 (50%)</td>
<td>5 (29.4%)</td>
<td>2 (66.7%)</td>
<td>8 (36.4%)</td>
</tr>
<tr>
<td>Low</td>
<td>1 (50%)</td>
<td>12 (70.6%)</td>
<td>1 (33.3%)</td>
<td>14 (63.6%)</td>
</tr>
<tr>
<td>Total</td>
<td>2 (100%)</td>
<td>17 (100%)</td>
<td>3 (100%)</td>
<td>22 (100%)</td>
</tr>
</tbody>
</table>

\[ P = 0.42 \text{ by } x^2\text{-test} \]