



Original Research

Distinct molecular phenotype and the potential prognostic value of immune prognostic index and tumor infiltrating lymphocytes in hepatoid adenocarcinoma of stomach

Muxing Kang^{a,b,1}, Xiaojing Ma^{c,1}, Jifei Shi^{a,d}, Guofeng Chen^a, Xiaoli Jin^a, Jun Wang^a, Lele Lin^a, Zhiwei Wu^a, Kaibo Chen^a, Jinghong Xu^c, Pintong Huang^{e,*}, Jian Chen^{a,*}

^a Department of Gastrointestinal Surgery, Second Affiliated Hospital, Zhejiang University School of Medicine, 88 Jiefang Road, 310009, Hangzhou, Zhejiang 310000, China

^b Key Laboratory of Cancer Prevention and Intervention, China National Ministry of Education, Cancer Institute, Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310000, China

^c Department of Pathology, Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310000, China

^d Department of General Surgery, The First Hospital of Pinghu, Jiaxing, Zhejiang 314200, China

^e Department of Radiology, Second Affiliated Hospital, Zhejiang University School of Medicine, 88 Jiefang Road, 310009, Hangzhou, Zhejiang 310000, China



ARTICLE INFO

Keywords:

Gastric cancer
Hepatoid adenocarcinoma of the stomach
Molecular typing
Predictive biomarkers

ABSTRACT

Hepatoid adenocarcinoma of the stomach (HAS) is a particular subtype of Gastric cancer (GC) with distinct pathological characteristics and genetic profile, but most HAS patients were received identical regimens as common GC. To date, only a few studies has been conducted to investigate the molecular characteristics of HAS, which may prevent the rational application of new anticancer strategies. To further obtain the genetic features and potential predictive and prognostic biomarkers of HAS, our current study evaluated the clinical implications of spectrum molecular markers in 36 surgical resection specimens. None Epstein-Barr virus (EBV) positive and/or micro-satellite instable high (MSI-h) tumors occurred in our study implies that the molecular classification of HAS should be mainly categorized into genomic stable (GS) and chromosomal instability (CIN) phenotypes, and wild type P53 status predicts better prognosis. More importantly, although the prognosis and clinical characteristics were independent of programmed cell death-ligand 1 (PD-L1), the presence of tumor infiltrating lymphocytes (TILs) still suggested that a portion of the enrolled HAS patients are potentially appropriate candidates for immune checkpoint blockade therapy. Additionally, the immune prognostic index (IPI) and derived neutrophil to lymphocyte ratio (dNLR) demonstrated their potential as reliable and economic indicators for predicting prognosis of HAS. We hope this first systematic evaluation will help in deciphering the molecular characterization and potential individualized regimens for this particular subtype of GC.

Introduction

Hepatoid adenocarcinoma of the stomach (HAS) is an exceptionally rare subtype that accounts for 0.38–1.6% of all gastric cancer (GC) with

worse prognosis [1–3]. HAS was first proposed by Ishikura et al [4]. This rare subtype of primary GC characterized by polygonal cells with a plentiful eosinophilic cytoplasm, which resembles of hepatocellular carcinoma, the diversified differentiation pattern further supports GC as

Abbreviations: ACRG, Asian Cancer Research Group; AFP, alpha-feta protein; AJCC, American Joint Committee on Cancer; CIN, chromosomal instability; CPS, combined positive score; DFS, disease free survival; dNLR, derived neutrophil to lymphocyte ratio; EBV, Epstein-Barr virus; EMT, epithelial to mesenchymal transition; FFPE, formalin-fixed and paraffin embedded; GC, gastric cancer; GS, genomic stable; HAS, hepatoid adenocarcinoma of the stomach; HE, hematoxylin-eosin; ICIs, immune checkpoint inhibitors; IHC, immunohistochemistry; IPI, immune prognostic index; MSI, microsatellite instability; LDH, lactate dehydrogenase; LIPI, lung immune prognostic index; MSS, microsatellite stable; OS, overall survival; PD-L1, programmed cell death-ligand 1; TCGA, The Cancer Genome Atlas; TILs, tumor infiltrating lymphocytes; TMB, tumor mutation burden; UICC, Union for International Cancer Control.

* Corresponding authors.

E-mail addresses: huangpintong@zju.edu.cn (P. Huang), zrchenjian@zju.edu.cn (J. Chen).

¹ These authors contributed equally to this work.

<https://doi.org/10.1016/j.tranon.2022.101380>

Received 28 January 2022; Accepted 19 February 2022

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a highly heterogeneous tumor. However, although HAS has more aggressive biological behaviors, different histogenetic identities and even distinct genetic profiles [5–8], most HAS patients are treated as common GC in clinical practice.

Recent advances in pathological mechanisms have fueled an upsurge in molecular typing and biomarker discovery in GC. The Cancer Genome Atlas (TCGA) [9] and Asian Cancer Research Group (ACRG), respectively, classified GC into four molecular subtypes [10], which are closely related to genetic contexts, treatment responses and prognoses. Likewise, novel biological markers, particularly microsatellite instability (MSI), programmed cell death ligand 1 (PD-L1), tumor infiltrating lymphocytes (TILs) and tumor mutation burden (TMB), can also effectively predict prognosis and even guide individualized treatment regimens for GC patients. Nevertheless, the molecular subtypes and novel biomarker expression patterns in HAS remain controversial and elusive.

Currently, advancements in the clinical application of immune checkpoint inhibitors (ICIs) drive the exploration of various molecular biomarkers that may reflect clinical efficacy and prognosis. Wang et al [5]. and Tsuruta et al [7]. attempted to identify the molecular characteristics and targets of HAS. Although these studies reported that most HAS patients had refractory malignancies with microsatellite stability (MSS) [5,7], favorable disease control from ICIs combined chemotherapy was observed [11]. These findings suggest that HAS is a genetically distinct subgroup of GC, and the reevaluation of molecular signatures and current protocols will contribute to a better understanding of the pathogenesis of HAS and development of precision therapies.

Accordingly, to further obtain the genetic features and potential predictive biomarkers of HAS, our current study evaluated the clinical implications of a spectrum of molecular markers in 36 surgical resection specimens. Among them, the tumor cell *in situ* MSI status, Epstein-Barr virus (EBV) status, combined positive score (CPS) of PD-L1 and POLE mutation were comprehensively assayed. Considering the decisive role of the immune microenvironment and peripheral proinflammatory status in tumor growth and treatment response, the clinical value of TILs and the immune prognostic index (IPI) were simultaneously assessed. We hope this first systematic evaluation will help in deciphering the molecular characterization and potential individualized regimens for this particular subtype of GC.

Materials and methods

Patients and case selection

36 Patients who underwent surgical resection for HAS, curative resection was performed in 31 patients, at the Second Affiliated Hospital of Zhejiang University School of Medicine between January 2008 and June 2018 were enrolled. Among them, 34 patients received fluorouracil-based adjuvant chemotherapy. All patients were followed up for at least three years after the operation. The definitive diagnosis of HAS was dependent on the histomorphological features and immunophenotypical evidence (immunohistochemistry staining of alpha-feta protein (AFP), glypican-3, SALL4, HepPar-1 and arginase-1). Tumor staging was determined according to the 8th edition of the guidelines of the Union for International Cancer Control/American Joint Committee on Cancer (UICC/AJCC) for the stomach. This retrospective study was approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine

DNA and RNA extraction

Cancer and matched normal samples were obtained as formalin-fixed and paraffin embedded (FFPE) tissue (five sections, each 7 µm thick). Genomic DNA was extracted using a DNA FFPE Tissue Kit (Amoy Diagnostics Co. Ltd). DNA purity and quantification were assessed using a NanoDrop 2000 UV–Vis spectrophotometer (NanoDrop products,

Wilmington, DE).

MSI status determination

The MSI Analysis kit (SinoMDgene Co. Ltd), was used for the detection of MSI. This kit allows the simultaneous evaluation of 6 fluorescently labelled MSI markers: NR-27, NR-24, NR-21, BAT-25, BAT-26 and MONO-27. PCR products were loaded into the ABI PRISM 3130XL Genetic Analyzer (Applied Biosystem). The data were analyzed using Gene Mapper software (Applied Biosystems), which automatically determined the actual size of the PCR products and the amount of fluorescent signal from electrophotography outputs. MSI was predicted by the presence of novel peaks in tumor tissue compared to the control. Instability in two or more microsatellite loci was categorized as MSI-high (MSI-h), and that in a single locus was categorized as MSI-low (MSI-l). The absence of MSI in all 6 markers and MSI-l were grouped as microsatellite stability (MSS) for further analyses following current guidelines.

POLE mutation determination

Mutations in the exonuclease domain of POLE (amino acids 268–471) were identified mainly in exons 9, 13, and 14. The primer sets used covered these regions and are described later in this subsection. Polymerase chain reaction (PCR) amplification and purification were performed according to the manufacturer's instructions (Shengggong, Shanghai), followed by Sanger sequencing. The primers used were as follows: POLE-Exon 9 forward, 5'-tgctattttgtcccccacag-3' and reverse, 5'-tacttcccagaagccacctg-3'; POLE-Exon 13 forward, 5'-tctgttctcattctctccag-3' and reverse 5'-cgggatgtgcttactgtg-3'; and POLE-Exon 14 forward 5'-tctggcttctctctcag-3' and reverse 5'-cga-caggacagataatgctca-3'. After confirming that mutations were somatic, the pathogenic impact of the mutation was annotated in three groups: hotspot mutations (P286R, V411L, S297F, A456P and S459F), POLE mutations with published high total mutational burden (TMB) (TMB>100Mb) (in our cohort A465V, D462Y, P436H), and POLE mutations of unknown significance (VUS). For the subsequent analysis, except where specifically stated, only the POLE hotspot mutations were defined as POLE mutated.

PD-L1 expression determination

Immunohistochemistry (IHC) was performed with a Ventana BenchMark ULTRA automated staining system according to the manufacturer's instructions using monoclonal antibodies against PD-L1 (clone E1L3N, 1:1000, Cell Signaling Technology) in 3 µm sections. The samples were processed in the automatic Ventana Benchmark Ultra platform using the OptiView Universal DAB detection kit and the OptiView Amplification kit. Tonsil samples were used as positive controls. Tris-buffered saline was used instead of primary antibody for negative controls. For PD-L1 analysis, the specimens were scored on the basis of the percentage of stained tumor cells (TCs) and tumor infiltrating immune cells (TIICs). PD-L1 positive cases were defined by the presence of at least 1% TCs or TIICs with membrane staining, regardless of the intensity. The highest score was selected if two or three cores from the same case exhibited different PD-L1 expression scores.

TP53 expression determination

Immunohistochemical analysis of TP53 expression in paraffin-embedded tissue sections was performed as previously described [12]. The serial sections were stained with hematoxylin-eosin (HE) and IHC (p53, DO-7, 1:200; Cell Signaling Technologies 48,818) in clinical HAS samples.

Immunohistochemical staining was evaluated and scored in a double blinded manner. Protein expression was assessed by using the sum of the

percentage positivity of stained TCs and the staining intensity. The percentage positivity was scored from 0 to 3, with 0 for < 10%, 1 for 10–30%, 2 for 31–50%, and 3 for > 50%. The staining intensity was classified follows: negative (score 0), weak (score 1), moderate (score 2) and strong (score 3). Subsequently, the expression was calculated as the value of percentage positivity score x staining intensity score, which ranged from 0 to 9. Drawing on published studies [13,14], the final expression level of p53 was defined as “wild type” (score 3–7) and “mutation status” (score 0–2 and 8–9).

EBV detection

EBV infection was studied by chromogenic *in situ* hybridization for EBV-encoded RNA (EBER-ISH, INFORM EBER probe, Ventana Medical Systems) using the same equipment as that described above, with enzymatic digestion (ISH protease) and the iViewBlue detection kit. The specimens from a patient with known EBV-positive gastric carcinoma were used as a positive control. A tumor was considered EBER-negative if EBER staining was undetected or was expressed only in benign-appearing lymphoid cells, and EBER-positive if the signal was localized to malignant epithelial cells.

TILs assessment

Since there is no current consensus on the morphologic evaluation of TILs in GC, the present TILs evaluation in HAS was based on a modified version of TILs scoring recommendations of the international TILs working group on breast cancer [15], and the reliability and repeatability of this scoring methodology was verified in GC [16,17]. Briefly, stromal TILs (str-TILs) was defined as the percentage of the tumor stromal area occupied by mononuclear inflammatory cells over the total intratumoral stromal area, and intratumoral TILs (itu-TILs) was defined as the percentage of the tumor epithelial nests that contained infiltrating lymphocytes. Regions of tissue necrosis, outside the tumor border and around normal gastric structures were excluded. All slides were scanned with an objective of $\times 40$ magnification, and the average number of TILs was assessed as a continuous semi-quantitative parameter. All cases were classified into high or low subgroup.

IPI determination

IPI score employed the criterion for lung immune prognostic index (LIPI) [18], which has been shown to be an effective prognostic marker as well as an ICIs treatment predictor for various solid tumors, including gastric cancer. Specifically, the IPI was calculated based on pretreatment levels of the derived neutrophil to lymphocyte ratio (dNLR) and lactate dehydrogenase (LDH). The dNLR was calculated as neutrophil count/(total leukocyte count – neutrophil count). The IPI was stratified into three groups: the good (score 0, LDH < normal and dNLR < 3), moderate (score 1, LDH \geq normal or dNLR \geq 3) and poor (score 2, LDH \geq normal and dNLR \geq 3).

Statistical analyses

The chi-square test or Fisher's exact test was performed as appropriate for categorical variables, and disease free survival (DFS) and overall survival (OS) were estimated by using the Kaplan-Meier method. DFS was calculated from the date of radical surgery to the date of death or adenocarcinoma relapse, whichever occurred first, in the 31 patients who underwent radical surgery. OS was defined as the time between surgery and death of any cause or last follow up in all 36 patients enrolled. A Cox model was used for multivariate analysis. All statistical analyses were performed using SPSS 17.0 software. $P < 0.05$ was regarded as statistically significant.

Results

Baseline clinicopathological features of 36 HAS patients

In the present study population, 31 patients underwent radical operation, and 5 patients received palliative resection. The baseline clinical and pathological characteristics are presented in **Supplementary Table 1**. Representative images of HAS, PD-L1 staining, itu-TILs and str-TILs are simultaneously shown in **Fig. 1**.

The data revealed that HAS had a higher prevalence in the elderly (> 60 years, 22/36, 61.1%) male (30/36, 83.33%) cohort. Most tumors (33, 91.67%) were located in the antrum and corpus of the stomach and only a few case tumors (3/36, 8.33%) were located in the gastroesophageal junction; among them, poorly differentiated tumors (32/36, 88.89%) with diameters less than 5 cm (26/36, 72.22%) represented the majority.

According to the 8th edition of the UICC and AJCC staging guidelines, 8 patients (22.22%) were pathological tumor-node-metastasis (pTNM) stage I/II, and 28 (77.78%) were stage III/IV. In particular, 29 patients (80.56%) had T3/4 tumors and 27 (75%) had lymph node metastasis. Not surprisingly, HAS was frequently accompanied by vascular invasion (29, 80.56%), and the liver was the most common site of the distant metastasis of HAS (3/4, 75%). AFP, a potential serum biomarker for HAS, was elevated in more than half of the patients (20/36, 55.56%), and 10 patients (10/36, 27.78%) had a serum AFP level \geq 200 ng/ml.

All present HAS cases were classified as the GS/CIN subtype, and the majority had TP53 mutations

The expression patterns of prognostic markers and ICIs curative effect predicted molecular targets are summarized in **Supplementary Table 2**. Nine patients (25%) had PD-L1 positive (CPS = 1) tumors, although notably, no patients had higher scores. It is also noteworthy that none of the HAS patients showed EBER positive reactions, MSI-h status or POLE mutations. This could indicate that HAS is a unique subtype of GC with specific genetic alterations, and most cases of HAS should be assigned to GS/CIN subtypes according to TCGA criteria. Considering the clinical practicality of ACRG classification, we further stratified the HAS patients into TP53 + (12/36, 33.33%) and TP53-types (24/36, 66.67%), which suggested that at least part of HAS patients should be assigned to the MSS/TP53+ subtype.

Regarding the expression level of TILs, positive str-TILs and itu-TILs were observed in 77.78% (28/36) and 86.11% (31/36) of the HAS patients, respectively. We used 20% as a cutoff limit in the present study, 27.78% (10/36) and 41.67% (15/36) of the patients were accordingly determined to have a high expression pattern of str-TILs or itu-TILs, respectively. Additionally, the IPI scores of 36.11% (13/36) patients were good (score 0), those for 41.67% (15/36) patients were moderate (score 1), and those for 22.22% (8/36) of the patients were poor (score 2).

TP53 rather than PD-L1 was significantly associated with clinicopathological characteristics, but neither serve as a prognostic factor for HAS

Statistical analysis revealed that the TP53 mutation cohort exhibited significantly more frequent lymph node metastases than the TP53 wild-type cohort (21 vs. 6, $P = 0.036$); correspondingly, the former group had a worse pathological stage ($P = 0.009$, **Supplementary Table 3**). Subsequently, survival analysis demonstrated a trend toward better OS and DFS for patients with a TP53 + status, but these improvements were not significant ($P = 0.15$ or $P = 0.32$, **Fig. 4.C. and F.**).

In contrast to the TP53 status, we did not observe any correlation between the CPS of PD-L1 and the clinicopathological characteristics of HAS (**Supplementary Table 4**). Although a portion of PD-L1 negative

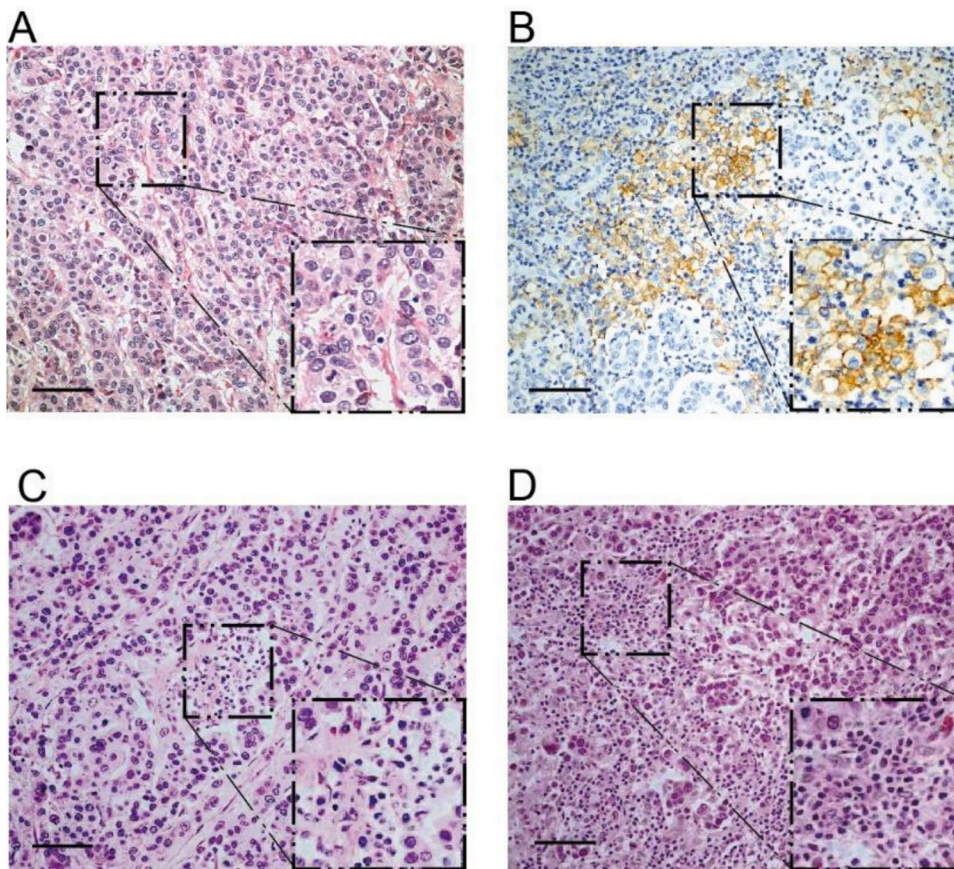


Fig. 1. Typical image of pathological staining. The representative photomicrographs of hematoxylin-eosin staining picture of HAS (A), the tumor composed of large polygonal eosinophilic hepatocellular carcinoma like cells, identically pathogenic manifestation was also observed in C. and D. Representatively immunohistochemical staining of PD-L1 positive expression in HAS (B). Typical hematoxylin and eosin staining of itu-TILs (C) and str-TILs (D). Original magnification $\times 200$, magnified picture $\times 400$, scale bar represents 50 μm .

patients tended to show better OS or DFS, the difference was not significant ($P = 0.62$ or $P = 0.93$, **Figs. 2.A. and 3.A.**).

itu-TILs rather than str-TILs was associated with TNM stage and prognosis of HAS

After stratification by TIL accumulation site, itu-TILs was not associated with tumor invasion depth, lymph node metastasis or vascular invasion, but lower itu-TILs was significantly associated with higher risk of lymph nodal metastasis and advanced TNM stages ($P = 0.019$, $P = 0.046$, **Supplementary Table 5**). Unexpectedly, the degree of str-TILs showed no association with any clinicopathologic characteristic (**Supplementary Table 6**). Correlation analysis further confirmed the positive relationship between advanced TNM stage and lower itu-TILs ($P = 0.03$, $F = 5.18$, $R^2 = 0.105$), but not with str-TILs ($P = 0.118$, $F = 2.572$, $R^2 = 0.07$).

Kaplan-Meier analysis explicitly showed that the higher itu-TILs group exhibited a more favorable prognosis ($P = 0.02$, **Fig. 2.B.**), the higher str-TILs group demonstrated a similar OS advantage but without significant difference ($P = 0.17$, **Fig. 2.C.**). Additionally, both the higher itu-TILs group and the higher str-TILs group showed a trend towards improved DFS, however this difference did not achieve statistical significance ($P = 0.09$, $P = 0.14$, respectively, **Fig. 3.B. and C.**).

Better IPI indicated improved prognosis in the HAS cohort

The index of systemic immune inflammation has been demonstrated to reflect the efficacy of ICIs and targeted therapy in a variety of human cancers [18,19]. We further performed an exploratory retrospective study in the HAS cohort. Fisher's exact analysis revealed that the cohort with a better IPI score may have a lower vascular invasion rate than the poor score group ($P = 0.044$, 1 sided or $P = 0.073$, 2 sided;

Supplementary Table 7).

Survival analysis further revealed that a better IPI score was associated with an improved OS (IPI score 0 vs. 1/2, $P = 0.02$; score 0 vs. 1, $P = 0.02$ score 0 vs. 2, $P = 0.04$; score 1 vs. 2, $P = 0.54$; **Fig. 2. D-F.**). A similar association between a better IPI score and a prolonged DFS was also found (IPI score 0 vs. 1/2, $P = 0.03$; score 0 vs. 1, $P = 0.03$; score 0 vs. 2, $P = 0.02$; score 1 vs. 2, $P = 0.42$; **Fig. 3. D-F.**)

Further stratified analysis indicated that compared with the plasma LDH concentration, the dNLR level was more relevant to clinicopathological characteristics and prognosis in HAS patients. Specifically, a better dNLR level was simultaneously associated with well differentiation ($P = 0.04$) and a decreased lymph node metastasis ratio ($P = 0.04$, 1 sided or $P = 0.055$, 2 sided; **Supplementary Table 8**). In contrast, the LDH concentration did not correlate with any parameter in the present analysis. As shown in **Fig. 4**, the estimated median survival time in the LDH high or normal group was 26 or 37 months, respectively, ($P = 0.29$), in contrast, the lower dNLR group (< 3) had obviously improved prognosis (mean overall survival time, 62.2 months vs. 23.2 months, $P = 0.01$) than patients with poor dNLR score (> 3). Furthermore, a lower dNLR score ($P = 0.03$), but not normal LDH concentration ($P = 0.33$), favored longer DFS in patients who received radical surgery.

Univariate and multivariate survival analysis of prognostic factors

At the time of the present study, 51.61% (16/31) of the patients had disease recurrence and 55.56% (20/36) of the patients died. Univariate and multivariate analyses were performed to evaluate the prognostic factors affecting DFS and OS.

Univariate analysis (**Table 1**) showed that lymph metastasis ($P = 0.038$), TNM stage ($P = 0.02$), itu-TILs ($P = 0.046$) and IPI score ($P = 0.039$) were significantly associated with OS, however, only TNM stage ($P = 0.035$) and IPI score ($P = 0.031$) were independent prognostic

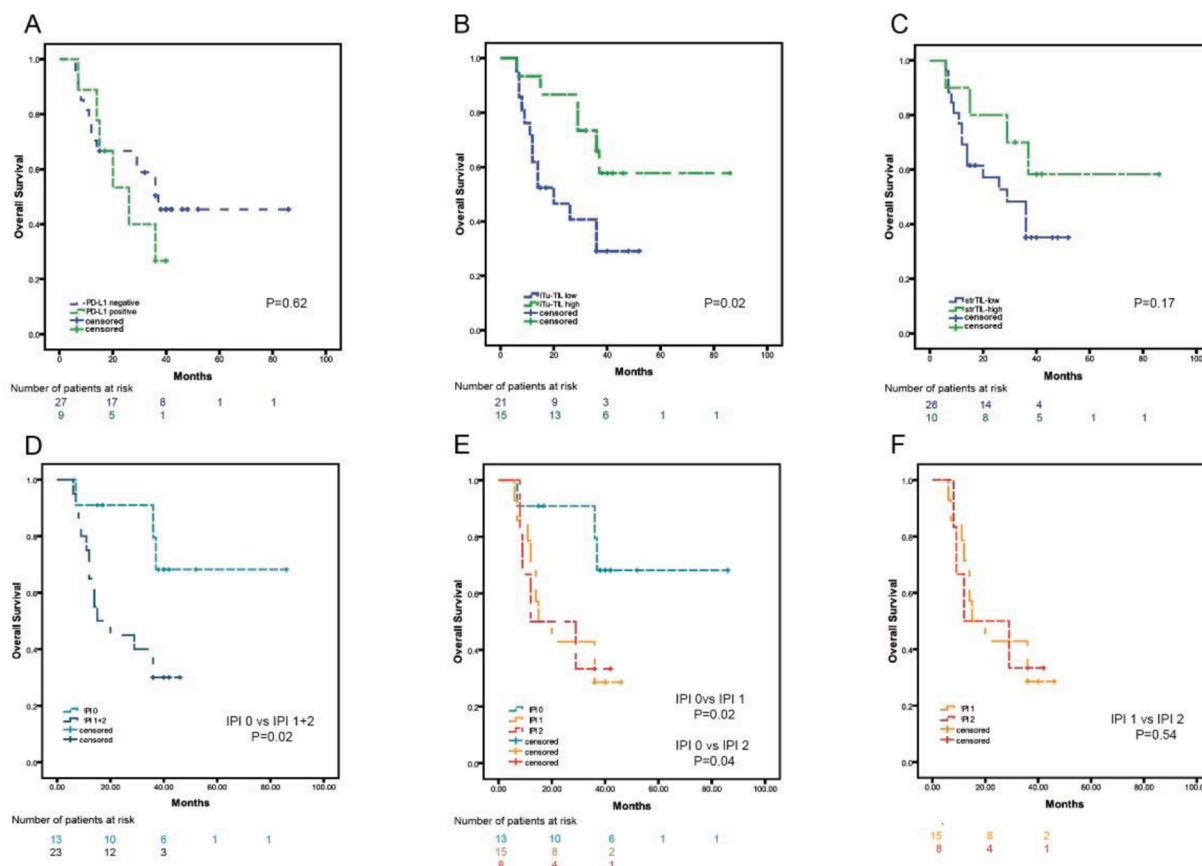


Fig. 2. Higher itu-TILs and lower IPI score, rather than PD-L1 and str-TILs, predicted improved overall survival in HAS patients although a minority of PD-L1 negative patients tended to show a better OS, yet without any statistical significance (A). Higher itu-TILs (B) rather than str-TILs (C) predicted a better OS. Similarly, better (lower) IPI score represented favorable prognosis (D), and the statistical difference was more evident with the IPI score decreasing (E and F). All 36 patients were included in the OS analysis.

factors for OS in Cox regression multivariate analysis.

IPI status ($P = 0.029$) and dNLR ($P = 0.01$) were closely associated with DFS in univariate analysis, however, the multivariate analysis failed to further confirm the statistical significance (Table 2).

Discussion

Despite the lack of comprehensive knowledge of the molecular cytogenetic features, most HAS patients receive regimens identical to those for common GC patients [1,2]. As such, the identification of predictive and prognostic factors to optimize the therapeutic protocol is therefore of crucial importance for patients suffering this highly heterogeneous malignancy. Checkpoint blockade immunotherapy, especially the PD-1/PD-L1 targeting regimen has recently revolutionized the treatment for GC [20,21], but the clinical features of HAS patients who could potentially benefit from this therapy remain controversial.

Although indicators including CPS, EBV, MSI-h and POLE/POLD1 mutations are very promising predictive markers for GC tumor response to ICIs therapy, the relationship between these markers and the therapeutic effect remains controversial [22,23]. Similar to a previous study [24], we did not find any influence of PD-L1 expression on the prognosis or clinical characteristics. Additionally, the positive status of EBV and MSI-h was extremely low [7] to meet the requirements for practical applications in the HAS cohort. A meaningfully retrospective study also suggested BRCA2 alteration as a potential biomarker associated with response to ICIs [25], however due to the interference of medication and the lack of prospective data [26], the specific roles and the screening value of BRCA1/2 in immunotherapy for GC patients remains uncharacterized. These current findings indicated that the screening of

biomarkers to predict the treatment response and selecting suitable candidates for ICIs have become a priority in the HAS cohort.

HAS is primarily categorized into the GS/CIN subtypes, and the majority of HAS patients have P53 mutation

Molecular classification and biomarker prediction are attractive approaches for revealing tumor pathological characteristics, and further facilitating the development of genome guided personalized therapy.

TCGA categorized gastric adenocarcinoma into EBV (9%), MSI (21%), genomically stable (GS, 20%) and chromosomal instability (CIN, 50%) phenotypes [9]. Additionally, the Asian Cancer Research Group (ACRG) classified GC into MSI (23%), MSS with intact p53 (MSS/TP53+, 36%), MSS with p53 mutations (MSS/TP53-, 26%) and MSS with epithelial-mesenchymal transition (MSS/EMT, 15%) [10]. These novel molecular classifications recognized the subtype with dismal prognosis (GS and MSS/EMT) and further detected the potential therapeutic targets.

Deviating from the proportions of each subtype in TCGA classification, all HAS patients (36/36, 100%) in the present study should be categorized into GS/CIN subtypes, and this proportion is significantly higher than that of common GC (69%) [9]. It is generally believed that cumulative epigenetic alterations lead to the more frequent occurrence of an MSI status in older patients, but we did not observe the same pattern in HAS patients. Compared with our research, previous findings showed that 94% of cases of HAS belonged to GS/CIN [7].

Notably, GS and CIN GC are generally considered to have poor therapeutic response to ICIs treatment, however, ICIs combined chemotherapy seems to function well in the clinical practice of HAS

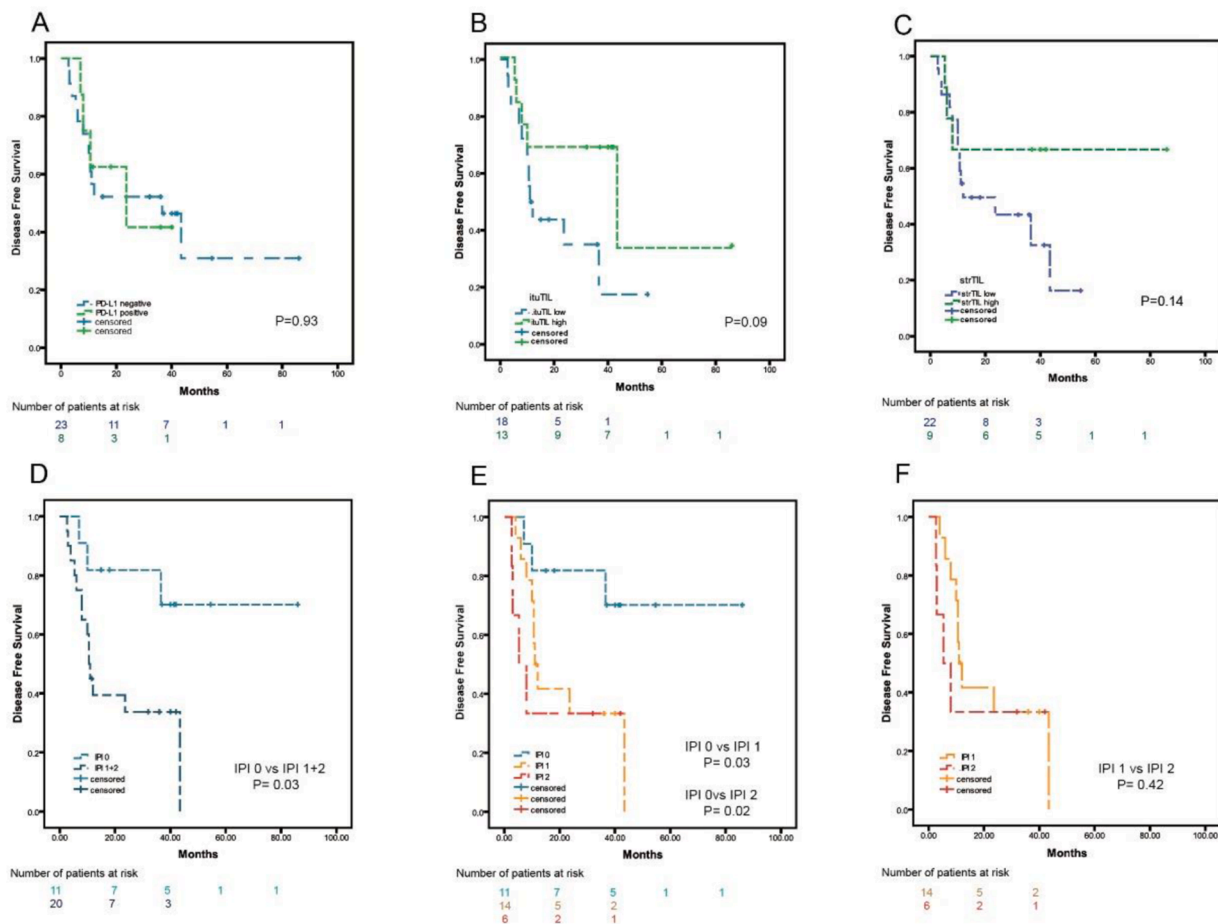


Fig. 3. Better IPI score, but not PD-L1 and TILs, predicted improved disease free survival in HAS patients PD-L1 expression was not associated with the DFS of HAS patients (A). Patients with higher itu-TILs and str-TILs denoted a possibility of better DFS, however, the trend did not reach statistical significance (B and C). Similarly with predictive role in OS, better (lower) IPI score was significantly associated with longer DFS (D), and the statistical difference was more meaningful with the IPI score decreasing (E and F). 31 patients received radical resection were included in the DFS analysis.

treatment [11]. A portion of HAS patients with TP53+ status may be one of the plausible explanations for this particular phenomenon, and these patients would be likely to exhibit a good response to ICIs therapy. The subtypes from TCGA or ACRG classification may have some overlap in molecular-pathological characteristics, for instance, the similarities between MSS/TP53+ and EBV, MSS/TP53- and CIN, MSS/EMT and GS subgroups [22,27].

Taking into consideration the clinical practicality of ACRG classification and the facilitation of P53 detection, we further stratified the HAS patients into TP53 + (33.33%, 12/36) and TP53- types (66.67%, 24/36), which suggested that at least some HAS patients should be assigned to the MSS/TP53+ subtype. GC patients with wild type TP53 generally have plentiful intratumoral lymphocyte infiltration [28], which is associated with favorable prognosis and increased ICIs sensitivity. Recently, an *in vivo* study further corroborated that the TP53 is a favorable condition for T cell infiltration and checkpoint therapy [29]. Likewise, the present study also observed an improved survival trend in patients with wild-type TP53 status, and the potential high mutation rate and TILs in MSS/TP53+ might be the underlying mechanisms for improved survival and further suggest the rationality of ICIs therapy in appropriated HAS patients.

itu-TILs can be used as prognostic indicators for HAS patients

TILs is a major manifestation of the host immune response against tumor progression, and it is considered as an immunotherapeutic signature that could guide personalized ICIs therapy [30]. The tumor

microenvironment has been divided into 4 types based on the presence of TILs and PD-L1 expression [31], GS and CIN GC were classified into II/III subtypes (cold non-inflamed microenvironment) with poor responders to PD-1/PD-L1 blockade therapy. From this perspective, only EBV and MSI GC have been widely recognized as immunogenic tumors and appropriate candidates for ICIs [32]. However, our study characterized partial HAS tumors that exhibited higher TILs status (41.67% with itu-TILs, and 27.78% with str-TILs), and higher itu-TILs was significantly correlated with better pTNM staging and prognosis. These results suggested that some HAS patients may benefit from ICIs therapy. Although TILs has demonstrated the capability to be an effective predictive parameter for ICIs in multiple tumor types [33,34], until now there has been no consensus on the specific assessment and exact predictive value for TILs in GC [35], and such limitations restrict their practical application in HAS patients. Therefore, further exploration for reliable and economic indicators to optimize the therapeutic protocol of HAS is warranted.

IPI and dNLR are convenient and economical prognostic markers for HAS patients

The systemic immune inflammation index has been confirmed as a prognostic factor in multiple cancers [22]. Inflammatory responses have been verified to participate in the initiation and progression of malignancies, and are even directly correlated with the status of circulating GC cells [36]. In the present study, although the IPI index only has a one sided statistical correlation with vascular invasion, significant and

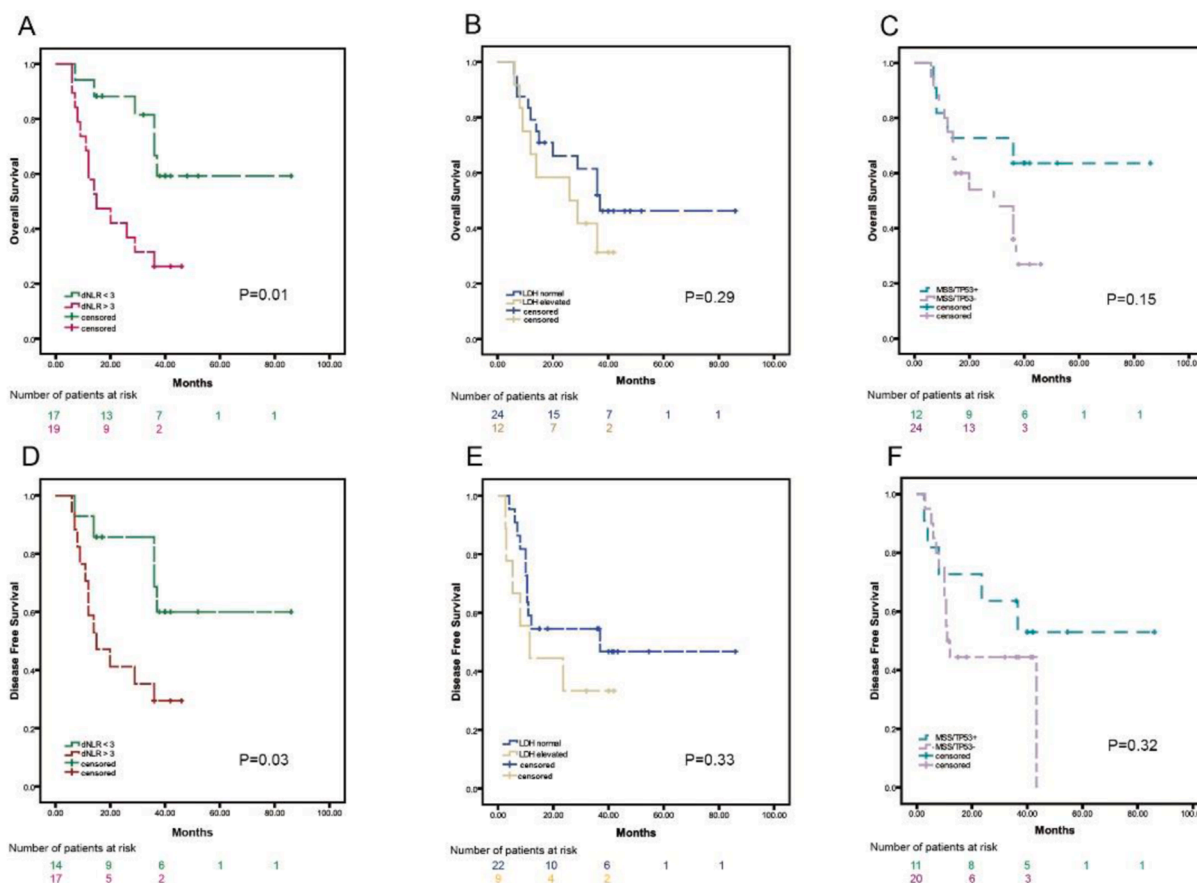


Fig. 4. dNLR, instead of LDH level and TP53 status, simultaneously associated with overall survival and disease free survival in HAS patients patients with normal dNLR (< 3) showed improved OS and DFS than the patients with elevated dNLR (> 3) (A and D). Although the patients with normal LDH level (B and E) and wild type TP53 (C and F) showed a certain degree of improvement in OS (B and C) and DFS (E and F), unfortunately, the differences did not research statistical significant. All 36 patients were included in the OS analysis, 31 patients received radical resection were included in the DFS analysis.

Table 1
Multivariate analysis for overall survival in all 36 HAS patients.

	Univariate analysis			Multivariate analysis		
	P	HR	95% CI	P	HR	95% CI
AGE ≤60 vs. > 60	0.942	1.038	0.382–2.818			
SEX M vs. F	0.130	0.209	0.028–1.587			
Differentiation	0.957	0.961	0.219–4.205			
Tumor location	0.334	0.481	0.109–2.120			
Tumor size	0.688	0.807	0.284–2.296			
> 5 cm vs. ≤ 5 cm						
T1 + 2 vs. T3 + 4	0.591	1.410	0.402–4.939			
N0 vs.N1–3	0.038	0.116	0.015–0.892			
TNM	0.020	5.846	1.326–25.765	0.035	5.730	1.129–29.091
III + IV VS. I + I						
Vascular invasion	0.116	0.027	0.001–2.433			
AFP	0.177	0.423	0.121–1.476			
PD-L1 expression	0.602	0.756	0.264–2.166			
itu-TILs	0.046	2.884	0.990–8.396			
str-TILs	0.320	1.778	0.572–5.526			
IPI score	0.039	0.264	0.075–0.933	0.031	0.218	0.055–0.867
dNLR	0.038	0.326	0.113–0.941			

inverse correlation with OS and DFS was observed, and the P value tended to increase with a higher IPI score. More meaningfully, the central component of the IPI, dNLR had a more significant statistical correlation with PFS and OS. As in a the previous study [18], we also considered that dNLR is more effective as a measure of immune system rather than LDH.

Neutrophils were not only conversely associated with OS, PFS and DFS [37], but also predicted a less favorable response to immunotherapy

in various tumors [38], and served as oncogenic factors that can even induce acquire immunosuppressive and tumor progression [39]. A higher dNLR is a potential hallmark of deficient cell mediated immunity and systemic inflammation, which may underlie the promotion of angiogenesis, DNA alteration and tumor proliferation [40]. Elevated baseline NLR was associated with worse ORR, PFS and OS in patients received PD-1/PD-L1 blockade therapy [41]. In a GC cohort, elevated NLR prior to and during treatment with nivolumab was associated with

Table 2

Multivariate analysis for disease free survival in 31 HAS patients who received radical surgery.

	Univariate analysis			Multivariate analysis		
	P	HR	95% CI	P	HR	95% CI
AGE ≤ 60 vs. > 60	0.767	0.864	0.328–2.272			
SEX	0.125	0.204	0.027–1.551			
Differentiation	0.481	2.072	0.273–15.710			
Tumor location	0.308	0.460	0.103–2.047			
Tumor size	0.585	0.747	0.263–2.124			
≤ 5 cm vs. > 5 cm						
T1 + 2 vs. T3 + 4	0.710	1.269	0.361–4.467			
N0 vs. N1–3	0.058	0.231	0.051–1.049			
TNM	0.152	2.289	0.738–7.101			
I + II VS. III + IV						
Vascular invasion	0.071	0.154	0.02–1.172			
AFP	0.354	0.588	0.191–1.807			
PD-L1 expression	0.929	1.053	0.338–3.287			
itu-TIL	0.106	0.413	0.141–1.206			
str-TIL	0.204	2.267	0.641–8.018			
IPI score	0.029	0.245	0.069–0.869			
dNLR	0.010	0.223	0.071–0.695			

worse prognosis [42]. In addition, there is argument that the combination score of local and host tumor immune response (TILs and NLR) could more precisely stratify patients with stage III colon cancer [43], but this potency have not been validated in the current and previous studies [44,45], the prognostic stratification approach merit further investigation in larger samples. The present analysis explicitly showed that the IPI and/or dNLR are potential predictive factors for HAS patients, and can even guide the development of individualized treatment plans.

In summary, the present study verified the molecular typing of HAS, and further explored the predictive and prognostic value of a series of immune checkpoint related indicators. As mentioned above, portion of the enrolled patients were potentially appropriate candidates for ICIs, although the vast majority of HAS patients should be categorized into the GS/CIN subtype. Moreover, the composite assessment of the expression status of TP53, TILs and IPI may demonstrate the potential of reliable and economic indicators to optimize therapeutic protocols for HAS patients. Naturally, more clinical and laboratory evidence with standardized methods and larger populations is needed to verify the above standpoint. The present study has contributed to the integrated knowledge and provided a new perspective for patient stratification in HAS cohorts.

CRediT authorship contribution statement

Muxing Kang: Conceptualization, Writing – original draft. **Xiaojing Ma:** Methodology, Formal analysis. **Jifei Shi:** Project administration. **Guofeng Chen:** Software. **Xiaoli Jin:** Resources. **Jun Wang:** Investigation. **Lele Lin:** Resources. **Zhiwei Wu:** Project administration. **Kaibo Chen:** Formal analysis. **Jinghong Xu:** Validation. **Pintong Huang:** Validation, Methodology. **Jian Chen:** Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Dr. Jian Chen was supported by Zhejiang Province Key Project of Research and Development (2019C03043) and Clinical Research Project of Zhejiang Medical Association (2018ZYC-A118). Dr. Muxing Kang was supported by Natural Science Foundation of Zhejiang Province (No. LY19H160042) and National Natural Science Foundation of China (No.

81301889). Dr. Pintong Huang was supported by the National Key R&D Program of China (2018YFC0115900), National Natural Science Foundation of China (82030048), Key Research and Development Program of Zhejiang Province (2019C03077).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101380.

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