Original Article

Analysis of prognostic germline polymorphisms in patients with advanced hepatocellular carcinoma

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Background: The prognosis of hepatocellular carcinoma (HCC) is influenced by both tumor and patient specific factors. Current therapies of advanced HCC target angiogenesis and immune evasion, however there are no clinically useful biomarkers to guide clinicians.

Methods: Our aim in this retrospective cohort study was to validate single nucleotide polymorphisms (SNPs) prognostic of outcome in advanced HCC from the literature, and to analyze exploratory SNPs chosen from evaluation of the HCC tumor immune microenvironment. Using a database of patients with HCC treated with sorafenib, blood samples were genotyped, clinical variables were retrospectively collected, and SNPs were analyzed for association with progression-free survival (PFS) and overall survival (OS). A subsequent analysis was conducted to determine if identified SNPs were prognostic in trans arterial chemoembolization (TACE) treated patients.

Results: Literature review identified 7 SNPs in vascular endothelial growth factor (VEGF), eNOS, angiopoietin 2 (ANGPT2) and vascular endothelial growth factor receptor 2 (VEGFR2), however none were externally validated in our dataset. Of the 35 exploratory immunomodulatory SNPs, the following were associated with PFS or OS: CCL2 C-C motif ligand 2 (CCL2) (rs1024611), interleukin-10 (*IL-10*) (rs1800896), cytotoxic T-lymphocyte antigen-4 (*CTLA-4*) (rs231775) and *NFKB1* (rs28362491).

Conclusions: SNPs identified by literature review to be prognostic in sorafenib treated patients with advanced HCC were not validated in our dataset. Our findings suggest potentially important prognostic implications of SNPs in VEGFR2, CCL2, IL-10, CTLA-4 and NFKB1 that deserve further study.

Keywords: Hepatocellular carcinoma (HCC); single nucleotide polymorphisms (SNPs); sorafenib; prognosis; biomarkers

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Introduction

Background

Hepatocellular carcinoma (HCC) is the 7th most common cause of cancer, and the 2nd leading cause of cancer deaths worldwide (1). The most important risk factor for HCC is liver cirrhosis, most often related to viral hepatitis. Additional risk factors include alcohol use, smoking, non-alcoholic fatty liver disease (NAFLD), and others.

HCC is a highly vascular tumor, underlining the importance of angiogenesis in disease progression. Research has shown angiogenesis in HCC is stimulated by direct production of angiogenic factors such as vascular endothelial growth factor (VEGF), angiopoietin-2 (Ang2), platelet-derived growth factor (PDGF) by malignant cells as well as infiltrating inflammatory cells (2). This occurs both due to hypoxia at the centre of the tumor, and through a constitutive production of angiogenic factors caused by mutations in tumor suppressor and oncogenes (2). High circulating levels of VEGF and high tumor microvessel density are associated with a more aggressive clinical course in HCC and worse survival (3).

The HCC microenvironment is composed of multiple cell types with immunomodulatory properties that can support carcinogenesis. A complex interplay between malignant cells and immune cells in the tumor microenvironment (TME) involving both immune stimulatory and immunosuppressive factors ultimately result in a milieu that prevents eradication of the tumor by the immune system. This signaling is mediated by a diverse set of molecules and cytokines (4) (*Figure 1*).

Multiple lines of research suggest crosstalk between angiogenesis and regulation of the immune microenvironment. Pro-angiogenic factors have a role in promoting an intratumoral immunosuppressive microenvironment. Signaling through VEGFR expressed on dendritic cells (DCs) has been shown to inhibit DC maturation (5). VEGF can promote the accumulation of immunosuppressive myeloid derived suppressor cells (MDSCs) in the TME (6). Moreover, VEGF can induce proliferation of other immunosuppressive cell types including Tregs (7) and tumor associated macrophages (TAMs). There is also evidence this relationship between angiogenesis and the immune system is reciprocal, with many immune cells fostering a niche that is supportive of angiogenesis. Both innate and adaptive immune cells within the TME can develop phenotypes that promote angiogenesis through several mechanisms. TAMs can support angiogenesis through the release of proangiogenic factors including VEGF and fibroblast growth factor-2 (FGF2) (8) as well as through breakdown of the extracellular matrix via the production of matrix metalloproteinases (MMPs) (9). Studies of immature myeloid cells including MDSC and DCs have demonstrated proangiogenic properties in the TME including the ability to release angiogenic factors such as VEGF as well as the ability to transform into TAM's or even endothelial cells (10). A recent successful clinical trial combined an anti-angiogenic agent (bevacizumab) with immune checkpoint inhibition (atezolizumab) with improvement in overall survival (OS), suggesting synergy between these pathways (11).

Sorafenib was the first systemic therapy to show convincing improvement of OS in advanced HCC in large, randomized trials. As a result of the landmark SHARP and Asia Pacific trials, sorafenib was widely adopted for the first line treatment of advanced HCC, however overall response rate (ORR) was low at 2%, and OS only improved from 7.9 to 10.7 months (12). Sorafenib is an orally administered, multi-serine/threonine and tyrosine kinase inhibitor (TKI) that targets VEGFR 1–3, PDGFR, FGFR1, KIT, RET, FLT3 and RAF pathways (13). The anticancer effects of sorafenib are thought to result both from inhibition of growth promoting intracellular signaling and from disruption of angiogenesis signaling in the TME. Only recently have additional systemic therapies shown

Highlight box

Key findings

- Single nucleotide polymorphisms (SNPs) prognostic in advanced hepatocellular carcinoma (HCC) were not validated in our dataset. Novel immunomodulatory SNPs in CCL2, IL-10, cytotoxic T-lymphocyte antigen-4 (CTLA-4) and NFKB1 were however found to be prognostic.

What is known and what is new?

- Sorafenib remains a key therapy for advanced HCC, despite recent novel treatment options. Observational data has suggested SNPs involved in genes in angiogenesis including VEGF, eNOS, ANGPT2 and VEGFR2 are prognostic in HCC.
- Prognostic SNPs from the literature were not externally validated. Exploratory SNPs within immunomodulatory pathways were significantly associated with prognosis in advanced HCC.

What is the implication, and what should change now?

- Identified prognostic immunomodulatory SNPs should be externally validated, and could provide guidance for treating clinicians and new targets for intervention.

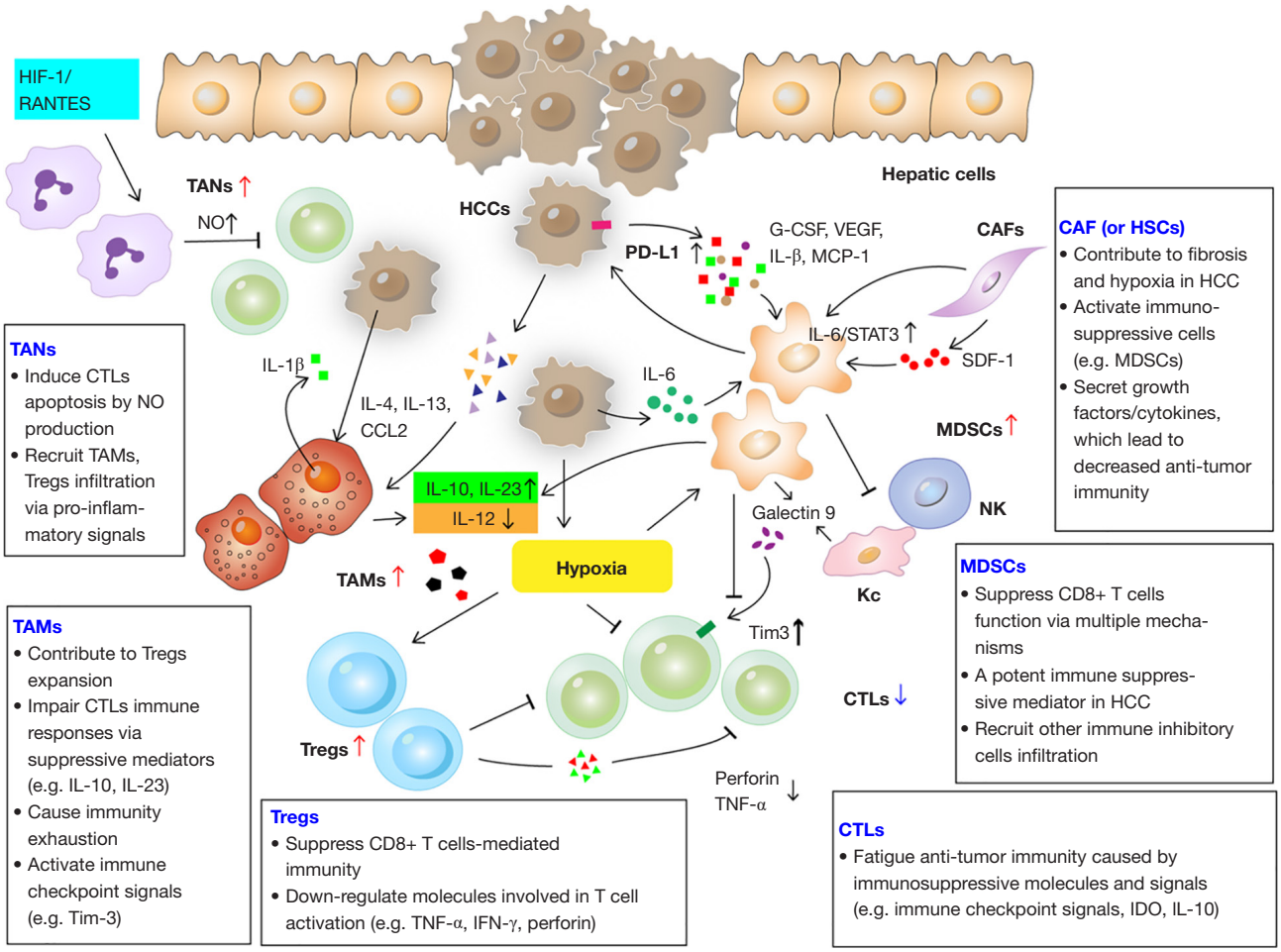

Figure 1 HCC tumor immune microenvironment (4). HCC, hepatocellular carcinoma; PD-L1, programmed death-ligand 1; TAN, tumor associated neutrophil; TAM, tumor associated macrophage; IL, interleukin; Treg, regulatory T-cell; CTLs, cytotoxic T lymphocytes; MDSCs, myeloid derived suppressor cells; Kc, kupffer cell; CAFs, cancer associated fibroblasts; HSC, hepatic stellate cell; IDO, inoleamine 2;3-dioxygenase; NO, nitric oxide; TNF, tumor necrosis factor; IFN, interferon.

convincing evidence of efficacy in advanced HCC, including additional oral TKIs, and atezolizumab, a programmed death-ligand 1 (PD-L1) inhibitor, in combination with bevacizumab. Despite these new options, many patients around the world will still receive treatment with sorafenib in a later line of therapy, or because of contraindications to atezolizumab or bevacizumab or because the high costs of these therapies make them unaffordable to most patients.

Rationale and knowledge gap

The prognosis of patients with HCC can be affected by several tumor specific and patient related factors. A systematic review by Tandon *et al.* identified 72 studies examining 79 prognostic factors in HCC, with almost 24,000 patients included. Five most common independent predictors of mortality were portal vein thrombosis, tumor size, Child-Pugh class, bilirubin and alpha-fetoprotein (AFP) (14). Validated prognostic biomarkers in HCC are lacking.

Single nucleotide polymorphisms (SNPs) are an attractive biomarker class due to their ease of measurement and ability to effect both tumor and host biology. In the context of cancer, SNPs have been most widely studied as risk factors for cancer development. In the context of HCC, Penha Mesquita *et al.* performed a systematic search of meta-analyses with revaluation by Bayesian calculations and found polymorphisms in *CCND1*, *CTLA4*, *EGF*,

IL6, *IL12A*, *KIF1B*, *MDM2*, *MICA*, *miR-499*, *MTHFR*, *PNPLA3*, *STAT4*, *TM6SF2* and *XPD* to be indicative of HCC risk (15). There are, however relatively sparse data existing on how SNPs influence prognosis in patients with existing cancer diagnoses. Polygenic risk scores are not currently used to inform prognosis in patients with established cancer diagnoses. There are however examples of individual SNPs found to have prognostic or predictive relevance for patient outcomes include *CYP3A4* in prostate cancer treated with androgen deprivation therapy, and *TPMT* in childhood leukemia receiving 6-mercaptopurine based treatment (16).

Objective

We aimed to review the literature to identify biomarkers with prognostic value in patients with advanced HCC, with a focus on SNPs. Patients with advanced HCC treated with Sorafenib were the main patient population of interest. Sorafenib was the standard of care therapy for advanced HCC at the time of study planning. From this initial literature search we aim to select SNPs with evidence for use as prognostic biomarkers, and externally validate them using a retrospective cohort of patients with advanced HCC at our institution. Furthermore, we will test exploratory immunomodulatory SNPs in our patient cohort. We aimed to analyze these SNPs in TACE treated patients because they also represent a group with incurable HCC. This allows for hypothesis generation as to whether the analyzed SNPs are prognostic in advanced HCC or more specifically predictive of sorafenib effect. We present this article in accordance with the STROBE reporting checklist (available at https://tgh.amegroups.com/article/view/10.21037/tgh-23-22/rc).

Methods

Structured literature review

A structured literature review of MEDLINE including Epub ahead of print, and EMBASE was performed to identify existing SNPs found to be prognostic in sorafenib treated patients with HCC. Refer to the search strategy document for details on the search algorithm.

Titles of potentially relevant studies results were selected from the list of search results. Following this, abstracts were reviewed for studies meeting eligibility criteria. The references of included studies were reviewed to find additional publications for inclusion. Studies identified in the structured review are listed (Table S1). The magnitude of the effect size of prognostic biomarkers was reported where available.

Eligibility criteria for study selection includes:

(I) Studies must have included patients with advanced/incurable HCC, who were treated with sorafenib;
(II) Included patients 18 years or older;
(III) Studies must have evaluated the prognostic or predictive ability of one or more SNPs against the clinical endpoints of either ORR, disease control rate, progression-free survival (PFS) or OS and have demonstrated a statistically significant association;
(IV) Published in the English language.

From this literature review, manuscripts of publications evaluating SNPs as prognostic biomarkers in sorafenib treated patients with HCC were reviewed. To avoid multiple comparisons, a maximum of 8 SNPs of interest were selected for external validation. Only SNPs with statistically significant findings in the original article were chosen. The disposition of studies identified in the literature review is presented in Figure S1. Additional factors forming the basis of SNP selection included strength of association [hazard ratio (HR), odds ratio (OR), relative risk (RR)], replication of SNP in multiple studies, biological plausibility, and study quality. Study quality was performed by assigning each manuscript a score using the REMARK framework (17). SNPs from studies with inadequate quality using the REMARK framework were not included for external validation.

Analysis of exploratory prognostic SNPs in patients with advanced HCC

Exploratory SNPs were selected using a candidate gene approach. Candidate genes were chosen that have a potential functional impact on either immunomodulation in the HCC TME, or impact on regulation of the immune system angiogenesis interface. To achieve this, a literature review was conducted to identify the immune cell types present in the HCC TME. Further, the literature was reviewed of cell types and signaling pathways involved in the interface between the immune system and angiogenesis. The full list of included cell type is as follows: TAMS, tumor associated neutrophils (TANs), mast cells, eosinophils, MDSCs, natural killer (NK), natural killer T cells (NKT), DCs, cytotoxic T lymphocytes (CTLs), Tregs, Kupffer, and endothelial cells. Genes critical to

the regulation of the above cell types, or genes encoding the signaling networks between the above cell types were chosen. Using this approach, the full list of analyzed genes includes: *ICAM1*, *VCAM1*, *EDNRA/B*, *EMAP2*, *Ang2*, *Tie2*, *IL1B*, *IL-A*, *IL2*, *IL4*, *IL5*, *IL6*, *IL8*, *IL10*, *IL12*, *IL13*, *IL17*, *IL18*, *M-CSF*, *CSFR1*, *MCP-1*, *SDF-1*, *SEMA3A*, *NRP1*, *GCSF*, *GM-CSF*, *STAT3*, *NFKB*, *IFNa*, *TNFa*, *TGFB*, *OncostatinM*, *CCL2*, *CCL3*, *CCL4*, *CCL5*, *CCL11*, *CCL15*, *CCL22*, *CCL28*, *CCR2*, *CXCL1*, *CXCL2*, *CXCL3*, *CXCL4*, *CXCL5*, *CXCL8*, *CXCL9*, *CXCL10*, *CXCL12*, *CXCL17*, *CXCR3*, *CXCR4*, *Bv8*, *iNOS*, *ARG1*, *IRF8*, *MMP1*, *MMP7*, *MMP9*, *MMP12*, *PD-L1*, *PD-1*, *CTLA-4*, *CD80*, *TIM3*, *LAG3*, *ICOS*, *FOXP3*, *GITR*, *Galectin9*, *CD25*.

From this list of candidate genes, another literature review was conducted to identify SNPs with a high level of evidence as being functionally active in the regulation of inflammation or angiogenesis. To achieve this, a search of OMIM and PubMed was conducted including each gene name, and the terms "SNP" and either "inflammation" or "angiogenesis". SNPs showing a statistically significant association with either laboratory or clinical markers of inflammation or angiogenesis in at least two independent studies were chosen for inclusion in the exploratory analysis.

This final list of exploratory SNPs can be found in the Table S2 and includes: rs10204525, rs1024611, rs1036199, rs1143627, rs1143634, rs11568818, rs11568821, rs16944, rs17561, rs17576, rs1799750, rs1799969, rs1799983, rs1800469, rs1800587, rs1800629, rs1800795, rs1800872, rs1800896, rs1870377, rs2010963, rs20541, rs2069762, rs2070744, rs2070874, rs2071559, rs2227306, rs2232365, rs2243250, rs2275913, rs2276109, rs2297518, rs231775, rs28362491, rs3024505, rs3212227, rs3761548, rs3816769, rs4073, rs4359426, rs4604006, rs5498, rs55633437.

Study patient population

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Institutional Review Board of Princess Margaret Cancer Centre (No. IRB00001258) and individual consent for this retrospective analysis was waived.

Patients for this study were drawn from a larger cohort of patients recruited to the Molecular Epidemiology of Hepatobiliary Tumors (HBT) Study at our institution. The population of interest included patients over the age of 18 with advanced HCC treated at our institution within the HBT database who have stored blood samples. One cohort included patients treated with sorafenib between October 1st 2008 and November 1st 2019, and another cohort included patients who received trans arterial chemoembolization (TACE) between January 1st 2002 and November 1st 2019. Starting dates reflect data availability in the HBT database. Patients must have had a diagnosis of HCC either through histologic confirmation or clinical diagnosis according to American Association for the Study of Liver Diseases (AASLD) guidelines (18). The decision for treatment was made by the treating clinician.

In the sorafenib cohort patients were selected with advanced HCC [Barcelona-Clinic Liver Cancer (BCLC)-C] or intermediate stage HCC (BCLC-B) refractory to or ineligible for curative therapies or chemoembolization. All patients received sorafenib at a dose of 400 mg po bid continuously with dose reductions applied as clinically indicated. In the TACE cohort patients were selected with intermediate stage HCC (BCLC-B), or early-stage HCC (BCLC-A) refractory to or ineligible for curative therapies alone. Patients with portal vein thrombus who were reviewed in a multidisciplinary conference and felt to be eligible for TACE were included. All patients received TACE with chemotherapeutic agents including doxorubicin, cisplatin or mitomycin, which were mixed with lipiodol, and emulsified with water-soluble contrast. Patient cohort details are outlined in the Consort diagram (*Figure 2*).

Additional eligibility criteria as follows:

Inclusion criteria:

- Banked blood sample available for SNP testing;
- Age 18 years or older;
- Date of blood collection January 1 2010–present;
- Received one of the following treatment modalities for HCC:
  - TACE;
  - Sorafenib.

Exclusion criteria:

- Mixed HCC/cholangiocarcinoma;
- Fibrolamellar HCC;
- Patients undergoing liver transplantation during treatment course.

Data collection

Clinical data was collected from electronic health records. Collected variables included demographic data, risk factors for HCC such as hepatitis virus infection status, prognostic variables, treatment data and outcomes. Outcome measures collected included date of disease progression, date of death, and requirement for dose reductions. The list and

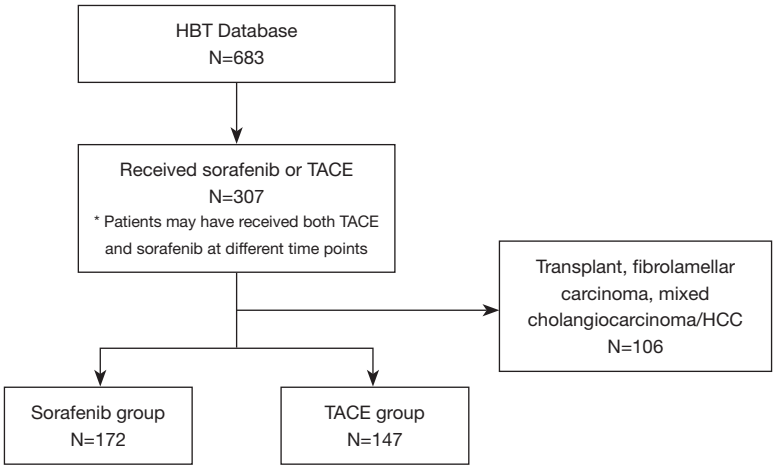

Figure 2 Consort diagram of patient cohort. HBT, Molecular Epidemiology of Hepatobiliary Tumors Study; TACE, transarterial chemoembolization; HCC, hepatocellular carcinoma.

definition of variables can be found in the data management document. Data are stored in password protected files. Existing data were utilized from previously collected sources including existing databases. For these patients, data quality was assessed by randomly selecting 10% of cases for secondary review.

Performance criteria

Selected biomarkers were evaluated on their prognostic value for patient outcomes.

Primary outcome:

- OS: defined as time from treatment (either TACE, or start of sorafenib) to death or censorship.

Secondary outcome:

- Real world PFS: defined as time from TACE or sorafenib until radiologic or physician-assessed disease progression or death. PFS can be a surrogate for OS, is clinically relevant to patients and often has improved power.

For patients with more than one treatment course of TACE, the first of such treatment was used for analysis of outcomes. For patients treated with multiple different treatment modalities throughout their illness course, outcomes were analyzed for each respective treatment modality (i.e., TACE or sorafenib).

Statistical analysis

Baseline characteristics were reported as medians and ranges for continuous variables and frequencies with percentages for categorical variables. Departure from Hardy-Weinberg equilibrium (HWE) were tested using the Pearson χ^2 test with a significant departure from HWE consider as P=5e–05. Linkage disequilibrium (LD) between SNPs were calculated as D' using the genetics package in RStudio.

Time-to-event data, such as PFS, were summarized using the Kaplan-Meier method using the starting date of sorafenib (or TACE) to the date of progression, death or last follow up. OS was estimated using the Kaplan-Meier method using the starting date of treatment to the date of death or last follow up.

The log-rank test was used to test univariable associations between clinical factors and OS, PFS. Necessary transformation of continuous variables was tested by examining martingale residuals. Complete case analysis was used to deal with missing data. Genotypes were coded using the additive model. A base clinical model was built to determine the independent predictive value of sequence variants. The base model included known prognostic factors determined from literature review including Child-Pugh score, T-stage, portal vein thrombus, AFP and neutrophil-lymphocyte ratio (NLR). Additional clinical factors were chosen via backward selection to retain in the clinical model with a significance level for staying (SLS) in the model cut-off of 0.2. SNPs were tested for prognostic ability using a log-rank test as well as likelihood ratio tests when added to the base clinical model. P values, HR and their 95% confidence intervals (CIs) for survival were reported. A two-sided type I error rate of 5% was used for

testing in this setting. The detectable HR for OS, assuming 80% statistical power and a two-sided significance level at 0.05, a sample size of 152 patients with an event rate of 106 (70% of patients) and the proportion of patients with a SNP of interest being 10%, is 0.327. A multivariable model including the base clinical variables and SNPs found to be significant on univariable testing was created. The proportional hazards assumption was tested for variables using Schonenfeld residuals. Statistical tests will be performed using R software.

Blood specimen analysis

Up to two tablespoons of blood was taken (30 cc or less) in purple top EDTA (whole blood), red top (serum), and yellow top Na-heparin (plasma) tubes. A research/laboratory technician stored the samples in a −70 ℃ freezer. Each peripheral blood sample DNA was extracted by ROCHE DNA Isolation Kit for Mammalian Blood (cat#11667327001). DNA yield and quality was controlled by Nanodrop. All the DNA samples were plated on four 96-well plates in 50 ng/μL, 10 μL well/sample. Sample plates were submitted to TCAG (https://www.tcag.ca) for MassARRAY genotype.

Samples were genotyped for 43 SNP positions in 2 (multiplex) panels using the MassARRAY® Analyzer 4 System (Agena Biosciences, San Diego, CA, USA) using iPlex Pro chemistry and analyzed using Typer 4.0 software. Briefly, each locus is amplified by polymerase chain reaction (PCR) and a third primer that flanks the polymorphism site is extended by one base (primer sequences are available upon request). The extension reaction products are analyzed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and correlated (by size) to the SNV and nucleotide observed. Commercial reference samples NA12878, NA12891, NA12892, NA18942 and NA18947 were genotyped with the study samples to validate each assay. Genotyping was performed blinded to outcomes.

Results

SNP genotyping results

Descriptions of polymorphisms selected for the external validation cohort and exploratory cohort are displayed in the Table S3. Genotypes are reported using the short form A (adenosine), C (cytosine), T (thymine), or G (guanine). All SNPs genotyped had an overall call rate >98%, except for one SNP rs1143627 which failed genotyping and was not included in the analysis (Table S4) for sample genotype and allele frequency. General population allele frequency was accessed via ALFA from dbSNP. Allele frequency of study participants was similar to the general population. All SNPs except for IL-4 (rs2070874), FOXP3 (rs2232365), IL-4 (rs2243250), FOXP3 (rs3761548) were in Hardy Weinberg equilibrium.

Patient characteristics-sorafenib treated HCC group

In total, 172 patients with HCC that were treated with sorafenib were included for analysis (*Table 1*). The median follow up was 10.6 months. The median age was 66.5 years, and 83% were male. The most common ethnicities were Caucasian and Asian. The etiology of cirrhosis in patients was hepatitis C virus (HCV) (36%), hepatitis B virus (HBV) (27%), alcohol (19%), and NAFLD (13%). Patients had frequently received prior therapy for HCC including resection, radiofrequency ablation (RFA), radiation and TACE. About 84% had multifocal HCC and half had portal venous tumor thrombus, half of patients had extrahepatic spread of tumor. And 93% had Child-Pugh A or B liver function. Dose reductions during sorafenib treatment were frequent at 80%. There were 106 deaths during follow-up. The median OS of all sorafenib treated patients was 15.4 months. There were 156 patients with progression or death during follow up, and the median PFS was 5.2 months.

Univariable analysis

Clinical variables

Univariable testing of clinical variables revealed a statistically significant association of only the Child-Pugh score with OS (median survival 19.2 months for Child-Pugh 5, 9.6 months for Child-Pugh 6, 11.9 months for Child-Pugh 7) and no clinical variables were significant predictors of PFS (Table S5).

Validation SNPs genotype analysis

Univariable analysis of SNPs selected for validation revealed only VEGFR2 (rs1870377) [minor allele frequency (MAF) =0.24] was statistically significant for prognosis of PFS (median 2.9 months AA, 5.3 months AT, 5.3 months TT, P=0.036 on log-rank testing, P=0.048 on nested likelihood ratio, HR 1.35 95% CI: 1.006–1.822), but not for OS (*Figure 3*, *Table 2*). No other SNPs in the validation cohort

Table 1 Clinical characteristics of patients treated with sorafenib

Clinical characteristic	Value (N=172)
Gender	
Male	143 [83]
Female	29 [17]
Age (years)	66.5 (17.6–87.2)
Ethnicity	
Asian/pacific islander	59 [34]
Caucasian	85 [49]
Black	4 [2]
Latino	1 [1]
Aboriginal	0
Other/mixed	10 [5]
Missing	13 [8]
Etiology	
HBV	44 [27]
HCV	61 [36]
Alcohol	32 [19]
NAFLD	22 [13]
Other	13 [5]
BCLC	
A	4 [2]
B	20 [12]
C	145 [84]
Missing	3 [2]
Serum AFP (ng/mL)	
≥200	77
<200	86
Missing	9
Prior therapy	
Surgical resection	30 [17]
RFA	52 [30]
TACE	58 [34]
Radiation	50 [29]
Transplant	0
Multifocal	
Yes	144 [84]
No	28 [16]
PVT	
Yes	88 [51]
No	84 [49]
Child-Pugh score	
A5	118 [69]
A6	41 [24]
B7	10 [6]
≥B8	3 [1]
Extrahepatic disease	
Yes	91 [53]
No	81 [47]
NLR*	
≥3	94
<3	71
Missing	7
Dose reduction of sorafenib	
Yes	138 [80]
No	34 [20]

Data are presented as n [%] or median (range). *, optimal cut-off based on the review by (19). HBV, hepatitis B virus; HCV, hepatitis C virus; NAFLD, non-alcoholic fatty liver disease; BCLC, Barcelona-Clinic Liver Cancer; AFP, alpha-fetoprotein; RFA, radiofrequency ablation; TACE, trans arterial chemoembolization; PVT, portal venous thrombosis; NLR, neutrophil-lymphocyte ratio.

were significant for PFS or OS.

Exploratory SNPs genotype analysis

Univariable analysis of exploratory genotypes revealed that CCL2 (rs1024611) (MAF =0.28) was a significant predictor of prognosis for OS (12.0 months AA, 20.8 months AG, 21.1 months GG, P=0.004) but not for PFS. The SNP IL-10 (rs1800896) (MAF =0.45) was prognostic for PFS

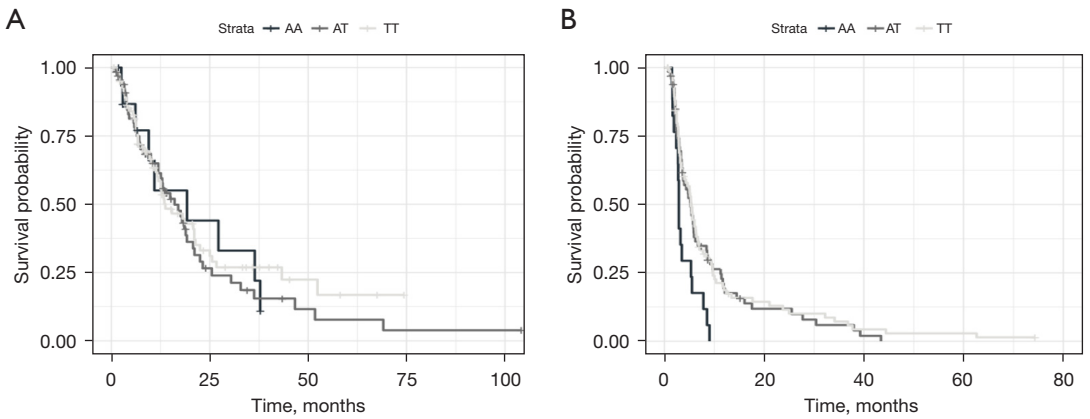

Figure 3 Kaplan-Meier curves for OS and PFS according to genotype for the VEGFR2 SNP rs1870377. (A) Univariable OS analysis: VEGFR2 (rs1870377). (B) Univariable PFS analysis: VEGFR2 (rs1870377). OS, overall survival; PFS, progression-free survival; VEGFR2, vascular endothelial growth factor receptor-2.

Table 2 Univariable analysis of validation SNPs-sorafenib treated patients

Gene	Genotypes (%)	Median PFS (months)				Median OS (months)			
		Wt.	Het.	Homo.	P	Wt.	Het.	Homo.	P
VEGFR2 rs1870377	AA/AT/TT (11/38/51)	5.3	5.3	2.9	*0.036, +0.048	13.7	16.1	19.2	*0.6, +0.61

*, log rank statistical test; +, nested LR statistical test. SNPs, single nucleotide polymorphisms; PFS, progression-free survival; OS, overall survival; wt, wild type; Het, heterozygous; Homo, homozygous; LR, likelihood ratio.

(9.6 months CC, 5.2 months CT, 4.8 months TT, P=0.0047) and for OS (25.5 months CC, 14.9 months CT, 13.0 months TT, P=0.014). CTLA-4 (rs231775) (MAF =0.37) was prognostic for PFS (5.5 months AA, 5.3 months AG, 3.0 months GG, P=0.035) but not for OS. NFKB1 (rs28362491) (MAF =0.42) was a predictor of PFS (5.7 months ATTG/ATTG, 5.1 months ATTG/DEL 3.6 months DEL/DEL, P=0.0087) and for OS (20.8 months, 12.9 months, 13.4 months, P=0.047) (*Figure 4*, *Table 3*).

Multivariable analysis SNPs sorafenib group

The clinical variables that were specified a-priori for inclusion in the multivariate model were gender, Child-Pugh score, T stage, portal vein tumor thrombus, AFP level and NLR. SNPs with a significant association on univariable testing were also included in the multivariable model with a final list of: VEGFR2 (rs1870377), CCL2 (rs1024611), IL-10 (rs1800896), CTLA4 (rs231775), and NFKB1 (rs28362491). Significant predictors of OS included T-stage, CCL2 (rs1024611), and IL-10 (rs1800896). Significant predictors of PFS included NFKB1 (rs28362491) (Table S6). None of the SNPs in the validation cohort were significant predictors of PFS or OS in the multivariate model. Testing of the proportional hazards assumption using Schoenfeld residuals revealed AFP was the only variable that violated the PH assumption.

TACE treated HCC group analysis

In total 147 patients with HCC that were treated with TACE were included for analysis (see Table S7 for description of clinical characteristics). The median survival of patients in the TACE database was 26.2 months, and the median PFS was 4.5 months.

Univariable analysis of clinical variables in the TACE treated cohort revealed an association of pre-treatment AFP level with OS. There was a significant association between age and BCLC stage with PFS (Table S8).

Univariable analysis SNPs-TACE Group

Among SNPs in the validation cohort, there was an association between NOS3 (rs2070744) and PFS (C/C

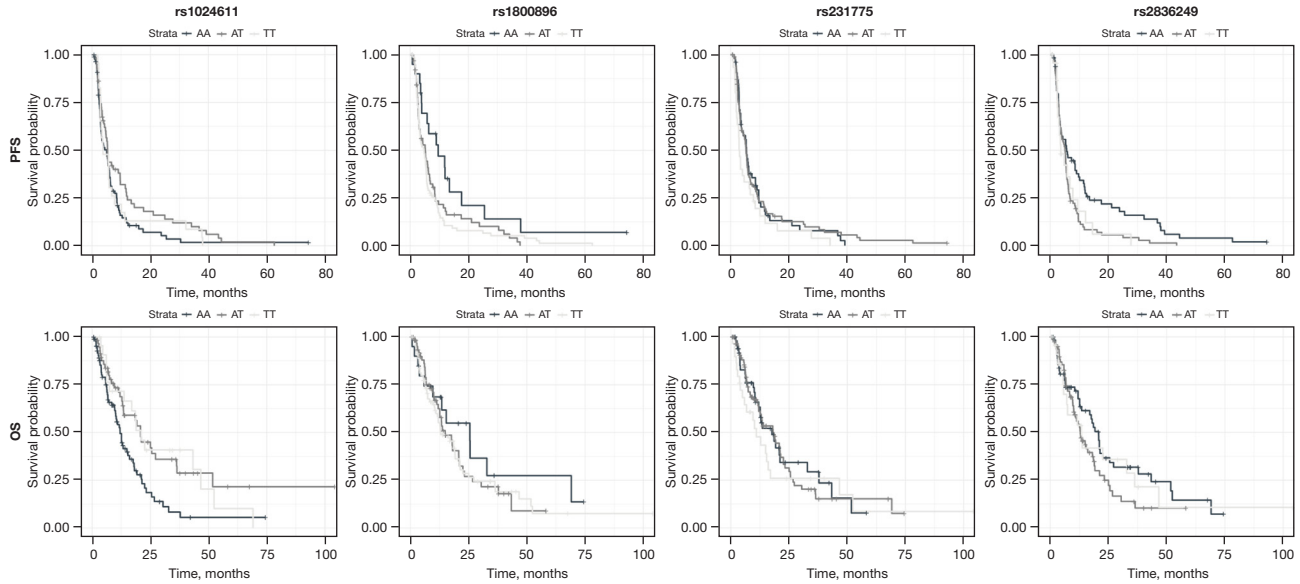

Figure 4 Univariable analysis of OS and PFS: exploratory SNPs. PFS, progression-free survival; OS, overall survival; SNPs, single nucleotide polymorphisms.

Table 3 Univariable analysis of exploratory SNPs-sorafenib treated patients

Gene	Genotypes (%)	Median PFS (months)				Median OS (months)			
		Wt.	Het.	Homo.	P	Wt.	Het.	Homo.	P
CCL2 rs1024611	AA/AG/GG (49/35/16)	5.0	5.3	3.8	*0.3, †0.47	12.0	20.8	21.1	*0.004, †0.065
IL-10 rs1800896	CC/CT/TT (12/39/49)	4.8	5.2	9.6	*0.02, †0.0047	13.0	14.9	25.5	*0.3, †0.014
CTLA4 rs231775	AA/AG/GG (31/49/20)	5.5	5.3	3.0	*0.2, †0.035	17.6	18.1	10.9	*0.4, †0.37
NFKB1 rs28362491	ATTG.ATTG/ATTG.DEL/DEL.DEL (40/46/15)	5.7	5.1	3.6	*0.01, †0.0087	20.8	12.9	13.4	*0.3, †0.047

*, log rank statistical test; †, nested LR statistical test. SNPs, single nucleotide polymorphisms; PFS, progression-free survival; OS, overall survival; wt, wild type; Het, heterozygous; Homo, homozygous; LR, likelihood ratio.

9 months, C/T 4.4 months, T/T 4 months, P=0.039), but not OS. There was an association with VEGFR2 (rs1870377) and OS on log rank testing but not on likelihood ratio testing compared to the base clinical model. Similarly, there was an association between CCL2 (rs1024611) and PFS on log rank testing but not on likelihood ratio testing compared to the base clinical model. TNF (rs1800629) was associated with OS (A/A 41 months, A/G 45 months, G/G 23.9 months P=0.007) but not PFS. IL-13 (rs20541) was associated with OS (G/G 25.5 months, A/G 30.7 months, A/A 45 months P=0.048) but not PFS. Finally, NFKB1 (rs28362491) was associated with OS (ATTG/ATTG 25.8 months, ATTG/DEL 24.3 months, DEL/DEL 28.8 months P=0.04 (Table S9).

Discussion

Key findings

Sorafenib remains an important treatment in a growing armamentarium for advanced HCC. However, response rates and improvements in survival are modest so selecting patients that may benefit would aid the decision making of clinicians. Prognostic factors used in the clinical setting have been incorporated into staging systems and include tumor size and spread, portal venous involvement, markers of liver function and patient functional status. Furthermore, AFP and NLR values have robust evidence for prognosis.

Thus far there have been limited clinically useful biomarkers for prognosis in patients with advanced HCC

beyond those above mentioned (Table S1). SNPs are an attractive class of biomarkers as they do not vary with time and are readily measurable from blood samples. Prognostically relevant SNPs may help stratify poorer risk patients, avoid over treatment, unnecessary side effects or help in determining more appropriate treatment strategies. Examples of SNPs with clinical utility in cancer treatment include the association of *XRCC1* codon 399 with prediction of platinum benefit in gastrointestinal, genitourinary and lung cancer. *XRCC1* is involved in DNA repair, a major therapeutic mechanism of platinum agents (20). In advanced prostate cancer, polymorphisms in *CYP19A1* effect testosterone metabolism and are associated with prognosis in androgen deprivation therapy treated patients (21).

We performed a literature review of studies examining the prognostic implications of SNPs in patients receiving sorafenib for advanced HCC and found data that the following SNPs were prognostic for clinically relevant outcomes: *VEGF*-C rs4604006, *VEGF*-A rs2010963, *eNOS* rs2070744, *eNOS* rs1799983, *ANGPT2* rs55633437, *VEGFR2* rs1870377, and *VEGFR2* rs2071559.

In the ePHAS study (22), 128 Italian patients were retrospectively analyzed. Included patients had advanced HCC and had received treatment with sorafenib. Polymorphisms of *eNOS* were analyzed for prognostic value under the hypothesis that sorafenib, by inhibiting VEGF-R would reduce nitric oxide production. Three *eNOS (NOS3)* polymorphisms were analyzed including –786T>C (rs2070744), VNTR 27bp 4a/b and +894G>T (rs1799983). Linkage disequilibrium was observed between *eNOS*-786 and *eNOS* VNTR (D'=0.85), and 4 haplotypes were observed (HT1-4). In the validation cohort, multivariate analysis revealed significant associations of eNOS-786 with PFS (HR 5.87), eNOS VNTR with OS (HR 7.04), eNOS+894 with OS (HR 11.95), and HT1 with PFS and OS (HR 11.17 and 7.03 respectively).

Marisi *et al.* (23), retrospectively studied 135 Italian patients with advanced HCC receiving sorafenib. Polymorphisms in *ANGPT2*, a signaling molecule critical in angiogenesis were tested for prognostic significance, as were the *eNOS* polymorphisms previously described in the ePHAS study. Eight *ANGPT2* SNPs were selected for inclusion. In multivariate analysis, *ANGPT2* rs55633437 and *NOS3* rs2070744 were found to be independent prognostic factors predicting PFS (HR 0.24; HR 6.32, respectively) and OS (HR 0.67; HR 5.48, respectively).

ALICE-1 (24) was a retrospective multicentre study that evaluated 148 patients with intermediate to advanced HCC who received treatment with sorafenib. The aim of the study was to assess SNPs in the VEGF and VEGFR genes to determine if they were prognostic in this group of patients. On multivariate analysis, rs2010963 (*VEGF*-A), rs4604006 (*VEGF*-C) were found to be independent prognostic factors for PFS (HR 0.25, 0.22 respectively) and OS (HR 0.28, 0.25 respectively).

A retrospective study was conducted by Zheng *et al.* (25) of 78 patients with advanced HCC treated with sorafenib in China. TagSNPs of *VEGFR2* with a MAF >0.1 were chosen by analysis of HapMap genotyping data. Four SNPs with that were nonsynonymous and another SNP in the promoter region of *VEGFR2* previously found to be functional were also included. On multivariate analysis, SNPs that were found to be prognostic included rs1870377 [time to progression (TTP): HR 0.68, OS: HR 0.35], and rs2071559 (OS: HR 2.25).

REMARK framework scoring demonstrated that each study had limited methodological deficiencies but were of an acceptable quality that the results could be tested as to their external validity (Appendices 1-4).

We aimed to external validate this list of SNPs to determine if these results were reproducible in our database of patients with advanced HCC. Furthermore, we aimed to analyze novel SNPs within immunomodulatory pathways to determine their prognostic significance in advanced HCC.

We failed to validate existing prognostic SNPs from the literature in our dataset. We did find that one SNP, rs1870377 was significantly associated with outcome in our dataset, however the direction of effect was opposite to that in the source paper. We did find that the immunomodulatory SNPs *CCL2* (rs1024611), *IL-10* (rs1800896), *CTLA*-4 (rs231775) and *NFKB1* (rs28362491) were prognostic in our dataset.

Strength and limitations

Sorafenib was the only approved therapy for advanced HCC during the dates of data collection for this study, and therefore is the agent all patients would have received if not deemed ineligible or if patients opted to pursue palliative care alone. In order to determine whether candidate biomarkers are predictive of sorafenib response instead of simply prognostic, they must be studied in a cohort of patients with HCC who are not exposed to sorafenib, which would not be possible by reviewing retrospective patient data. As an exploratory analysis, we evaluated candidate biomarkers in patients with HCC treated with TACE to

determine whether they might be prognostic in this group also. An exploratory analysis of the interaction between biomarker status and treatment modality will be performed to determine whether tested biomarkers gave different results in patients not treated with sorafenib. This is a limitation because patients in the control group will have earlier stage, lower volume disease with a better prognosis and so comparisons will be indirect, although these potential confounders will be included in the statistical model to control for this where possible.

Comparison with similar research and explanation of findings

Of the SNPs selected for external validation, only VEGFR2 (rs1870377) was significantly associated with outcome in our dataset. VEGFR2 (rs1870377) was associated with PFS (median 2.9 months AA, 5.3 months AT, 5.3 months TT, P=0.048, HR 1.35, 95% CI: 1.006–1.822) but not OS. In the paper by Zheng *et al.* (25) from which the SNP was selected, VEGFR2 (rs1870377) AA was prognostic for TTP and OS but with a HR less than 1 suggesting a protective effect which was opposite in direction to the effect found in our dataset raising doubts about the validity of the prognostic value of the variant. rs1870377 T->A results in a missense mutation resulting in an amino acid alteration in the extracellular domain of VEGFR-2, 472H>Q which has been shown to decrease the affinity of VEGFR-2 for its ligand VEGF (26). In a study by Zhu *et al.* (27) *VEGFR-2* SNPs were studied as prognostic biomarkers in patients with resected gastric cancer and found rs1870377 was prognostic (AA *vs.* AT, HR 1.69). Zhuo *et al.* (28) found that in advanced gastric cancer patients rs1870377 was associated with OS (AA/AT *vs.* TT HR 2.04). Interestingly, there have been other studies showing a prognostic implication of rs1870377 in patients with advanced cancer receiving treatment with anti-VEGF therapies. Maeng *et al.* (29) found that in patients with advanced gastric or biliary cancers receiving sunitinib, rs1870377 was associated with prognosis AA *vs.* AT/TT HR 2.27). Gal *et al.* (30) studied patients with metastatic breast cancer treated with fist line paclitaxel and bevacizumab and round rs1870377 was prognostic for OS (AA/AT *vs.* TT HR 1.69). These results are congruent with the findings of our study suggesting this SNP may be a relevant biomarker in patients undergoing anti-VEGF therapies.

Significant basic science and clinical research has suggested an important role of the tumor immune microenvironment, and its crosstalk with angiogenesis in the prognosis of HCC. Therefore, an exploratory analysis of immunomodulatory SNPs was carried out to determine their prognostic value in patients with advanced HCC treated with sorafenib (Table S2). SNPs in four immunomodulatory genes were found to be prognostic in our dataset, including *CCL2*, *IL-10*, *CTLA-4* and *NFKB1*.

An association was found for the *CCL2* SNP rs1024611 for OS (HR 0.67, G *vs.* A) with no association for PFS. Functionally, rs1024611 is located in the 5' UTR of *CCL2*. rs1024611 influences the expression of *CCL2* through allelic expression imbalance with preferential expression of the G allele. An *in vitro* study found A>G lead to lower levels of CCL2 in a dose dependent manner (31). Tse *et al.* (32) found an association between the rs1024611 GG genotype and *CCL2* expression in nasopharyngeal tumors and with tumor macrophage infiltration. rs1024611 was also associated with the development of distant metastasis in patients treated with radiotherapy. Patients with the AA or AG genotype had a worse distant metastasis free survival (DMFS) compared to the GG genotype (HR 2.21, or 2.23 respectively).

An association was found with the *CTLA-4* SNP rs231775 and PFS (HR 1.17 G *vs.* A) but not with OS. Functionally, rs231775 results in a missense mutation in *CTLA*-4, with the G allele leading to decreased expression at the cell surface (33). Liu *et al.* (33) studied rs231775 in patients with advanced RCC patients receiving sunitinib and found an association with OS (GG *vs.* AG/AA HR 0.83) however directionally the effect was opposite to that seen in our analysis, with patients with the GG genotype associated with improved prognosis. Similarly, Machado-Rugolo *et al.* (34) found an association of rs231775 with prognosis in patients with advanced NSCLC but with an opposite direction of effect as found in our analysis.

An association was found for the *IL-10* rs1800896 for both OS (HR 0.87 C *vs.* T) and PFS (HR 0.77 C *vs.* T). Functionally, rs1800896 has two potential functional effects. On the plus strand, rs1800896 is upstream of *IL-10*, with the G allele being associated with increased levels of IL-10 mRNA (35). On the minus strand, rs1800896 is an intronic variant within *IL-19*. rs1800896 does not have well documented prognostic implications in other cancer types in the literature (36).

An association was found with the *NFKB1* SNP, rs28362491 and OS (HR 1.14 DEL *vs.* ATTG) as well as with PFS (HR 1.35 DEL *vs.* ATTG). Functionally encodes for *NFKB1* insertion/deletion in the promoter region, and

prior work has shown the deletion variant to be associated with less promoter activity (37). The literature has not described an association between this polymorphism and prognosis in other cancer types.

SNPs were tested for prognostic value in patients with advanced HCC treated with TACE. Of the SNPs found to be prognostic in sorafenib treated patients, only NFKB1 rs28362491 was also found to be prognostic for TACE treated patients but the HR directions was opposite to that observed in the sorafenib treated patients. This finding can be considered hypothesis generating that the identified SNPs in the sorafenib treated patients may be predictive to sorafenib effect, however this would require confirmation, ideally in a trial in which patients were randomized to either receive or not receive sorafenib.

Implications and actions needed

Although our dataset did not validate any of the selected SNPs, VEGFR2 (rs1870377) deserves further study in an independent dataset as a prognostic factor because our results corroborate the effect seen in studies other tumor sites. Furthermore, *CCL2* (rs1024611), *IL-10* (rs1800896), *CTLA*-4 (rs231775) and *NFKB1* (rs28362491) should be validated in external datasets and in patients treated with modern therapies. If confirmed, these SNPs could be retrospectively evaluated in randomized trials of sorafenib to determine if they are only prognostic or may be predictive of response to sorafenib. If validated these SNPs could also be incorporated into existing prognostic scoring systems and inform basic science investigations to better understand the HCC TME.

Conclusions

In summary, HCC is a global, highly prevalent disease with poor treatment outcomes and limited biomarkers available to guide clinicians on prognosis. We failed to validate prognostic SNPs from the literature in our dataset. We did find that one SNP, VEGFR2 (rs1870377) was significantly associated with outcome in our dataset, however the direction of effect was opposite to that in the source paper. Further literature review revealed that VEGFR2 (rs1870377) is prognostic in other cancer types treated with anti-VEGF therapies with a similar direction of effect as in our data. These findings underscore the difficulty repeating SNP results in observational studies given the potential for spurious associations when multiple variants are tested, effect sizes are not large or when sample sizes are low. We identified SNPs with functional impact on immune signaling in the HCC TME and tested their prognostic significance in sorafenib treated patients. We found that *CCL2* (rs1024611), *IL-10* (rs1800896), *CTLA-4* (rs231775) and *NFKB1* (rs28362491) were prognostic in our dataset. Finally, each of the validation and exploratory SNPs were tested in TACE treated patients and were not found to be prognostic, indicating a possible interaction effect with treatment modality.

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Footnote

Reporting Checklist: The authors have completed the STROBE reporting checklist. Available at https://tgh.amegroups.com/article/view/10.21037/tgh-23-22/rc

Data Sharing Statement: Available at https://tgh.amegroups.com/article/view/10.21037/tgh-23-22/dss

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related

to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Institutional Review Board of Princess Margaret Cancer Centre (No. IRB00001258) and individual consent for this retrospective analysis was waived.

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Supplementary

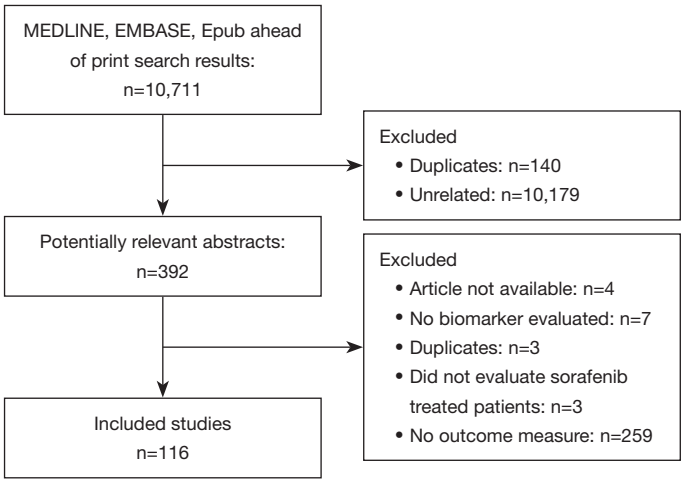

Figure S1 Consort diagram of literature review. Breakdown of the numbers of study results from initial literature search as well as number of studies excluded after reviewing abstracts and full papers.

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Table S1 Studies of biomarkers prognostic or predictive of sorafenib in advanced HCC

Article	Study description	N	Tested biomarker(s)	Results	Comparator non-sorafenib cohort?
Next generation sequencing					
(38)	Cohort study	13	FGFR ¼ amplification	FGFR3/4 amplification predicts for response	N
(39)	Cohort study	127	341 cancer associated genes	PI3K-mTOR pathway alterations were associated with reduced DCR, PFS, OS	Y-Immune CPI
(40)	Cohort study	46	40 genes for DNA and RNA sequencing	Average number of oncogene mutations predicts disease control, RNA expression of *TGFa*, *PECAM1*, and *NRG1* predicts PFS	N
(41)	Gene database analysis		1,319 differentially expressed genes	8 hub genes for sorafenib resistant phenotype kininogen 1, vascular cell adhesion molecule 1, apolipoprotein C3, alpha 2-HS glycoprotein, erb-b2 receptor tyrosine kinase 2, secreted protein acidic and cysteine rich, vitronectin and vimentin	N
(42)	Cohort study	45	FGFR genetic alterations	FGF19 copy number gain predicts CR	N
(43)	Cohort study	42	Genomic profiling of 381 cancer associated genes	Cell cycle gene aberrations predicts lack of response	N
(44)	Cohort study	47	mRNA expression of the CSC genes *EpCAM*, *CD13*, *CK8*, *CD24*, *CD44*, *CD90*, *CD133*, *SALL4*, *ALDH1A1*, *ALB*, and *AFP*	High CD133/CD90 expression predicts OS (HR 2.97)	N
(45)	Cohort study	151	Plasma ctDNA, genome wide CNA, VEGFA amplification	ctDNA level predicts OS (HR 2.5), CNA predicts OS (HR 1.85)	N
(46)	Case report	1	Tumor neoantigens were identified using whole exome sequencing	mutated IL-1β^{P208F} peptide and two additional neoepitopes from HELZ2^{G815A} and MLL2^{A3498V}	N
Tissue IHC					
(47)	Cohort study	39	IHC for p-Jun, p-JNK, CD133	High levels of p-Jun, p-JNK, CD133 associated with worse response	N
(48)	Cohort study	93. 65 received sorafenib	VEGFR-2, PDGFR-β, and c-Met	Low PDGFR-B associated with improved OS, high c-MET associated with improved PFS	N
(49)	Phase 2 trial	137	Tumor IHC pERK, blood cell-RNA microarray analysis	Higher pERK associated with longer TTP. No HR given. 18 genes in blood predicted 'progressors'	N
(50)	Cohort study	73	Ki67, CK19, glutamine synthetase, VEGF, VCP, pERK	Ki67 >20, CK19, VCP associated with OS	N
(51)	Cohort study	54	pERK, S6K, VEGFR2, PTEN	pERKs3 predicts OS (HR 1.504)	N
(52)	Cohort study	50	p-c-Jun	p-c-Jun high predicts OS (HR 2.3)	N
(53)	Cohort study	39	OCT-1	Tumor cell IHC staining for OCT-1 predicts improved OS. No effect measure reported	N
(54)	Phase 3 trials	77	β-catenin glutamine synthetase (GS), phosphorylated extracellular signal regulated kinase (pERK), phosphorylated v-akt murine thymoma viral oncogene homolog (pAKT) and FLK-1/KDR/VEGFR-2	pERK predicts OS (HR 2.09), VEGFR-2 predicts OS (HR 2.28)	N
(55)	Cohort study	35	VEGFR1, 2 expression	Lack of VEGFR1,2 predicts poor OS	N
(56)	Cohort study	44	Mcl-1, activated/phosphorylated extracellular signal-regulated kinase (pERK) 1/2, and activated/phosphorylated AKT (pAKT MYC and MET by FISH	pERK predicts OS (HR 1.013), MCL-1 predicts OS (HR 1.016)	N
(57)	Cohort study	83	HTATIP2, microvessel density	High HTATIP2 and low microvessel density predicts poor OS	N
(58)	Cohort study	41	CXCR4 expression	High CXCR4 expression predicts better OS	N
(59)	Cohort study	94	EDN1 expression	High EDN1 predicts OS (HR 2.374)	N
Circulating tumor cells					
(60)	Cohort study	59	Circulating tumor IHC p-ERK, p-AKT	Patients with pERK$^+$/pAkt$^-$ CTC Had improved DCR and PFS (HR 9.4)	N
Blood counts					
(61)	Phase 3 trial	170	Platelet count	Platelet count >150 predicts worse TTP HR 1.56	N
(62)	Cohort study	145	Baseline neutrophil lymphocyte ratio	NLRs4 HR 1.73 for OS	N
(63)	Cohort study	43	PBMC ROS and pERK	PBMC ROS and pERK predicts response	N- patients also received octreotide LAR
(64)	Cohort study	56	Systemic immune-inflammation index, NLR, PLR	SIIs360 HR 2.99 for OS, NLRs3 HR 2.36 for OS	N
(65)	Cohort study	161	neutrophil-to lymphocyte ratio (NLR), the derived NLR, the platelet-to-lymphocyte ratio (PLR), the monocyte-to-lymphocyte ratio (MLR), the prognostic nutritional index (PNI) and the systemic-immune inflammation index (SII	systemic immune-inflammation index (SII) ≥600×10^9 was independent predictor of OS (HR 1.72)	N
(66)	Cohort study	105	NLR	NLR >3.5 predictive of OS (HR 0.5), AFP <1030 ng/mL predictive of OS (HR 1.93)	N
(67)	Cohort study	82	NLR	NLR decline predicts PFS and OS (HR 0.479)	N
(68)	Cohort study	442	NLR, RDW	NLR predicts OS (HR 1.218), and RDW predicts OS (HR 1.234)	N
(69)	Cohort study	19	PD-1 Tcells, Tregs, MDSCs, cytokines	OS predicted by decrease in CD4/CD8+ PD-1+ Tcells and Foxp3+ Tregs	N
(70)	Cohort study	154	NLR	NLR >2.3 predicts OS (HR 1.72)	N
(71)	Phase 2	40	CEPs, CEC's	CEP predicts OS (HR 2.512) and PFS	N- Sorafenib+ metronomic chemo
(72)	Cohort study	142	MLR	MLR >0.35 predicts OS (HR 0.445), AFP predicts OS (HR 0.445)	
Alpha-fetoprotein					
(73)	Phase 2 trial	544	AFP	AFP <200 had HR 0.679 for OS on multivariate testing	N
(74)	Cohort study	214	AFP, NLR	AFPs7 ng/mL HR for OS 1.64	N
(75)	Phase 2 trial	1130	AFP	Log AFP ng/mL HR 1.087 for OS	N
(76)	Cohort study	320	AFP	AFP reduction of >20% at 3 months predictive of OS HR 0.38	N
(77)	Cohort study	225	AFP	AFP >456 predicts OS (HR 1.76)	N
(78)	Cohort study	254	AFP	AFP >200 ng/mL predicts OS (HR 1.45)	N
Circulating protein					
(79)	Phase 2 trial of sorafenib plus Trebananib	60	Ang-2	Ang-2 >5,700 ng/mL had HR 2.43 for OS	N
(80)	Cohort study	101	IGF-1	Addition of IGF-1 to CP scoring system improved prediction of OS and PFS	N
(81)	Cohort study	23	Chromogranin-A, VEGF	chromogranin A and VEGF were inversely correlated with response. No effect measure given	N
(82)	Analysis of Sharp & AP trials	827	Clinical variables, albumin, AFP, ALP	HCV, Low NLR showed significant interaction with treatment	Y-placebo
(83)	Cohort study	62	VEGF-A, b FGF, sVEGFR2, Ang2, SDF1, VEGF-C, IL-6, IL-8, AFP, HGF, TSP1, BMP9	Ang2, sVEGFR2, IL-6, IL-8, AFP associated with OS	N
(84)	Cohort study	30	IGF-1	Baseline IGF-1 level predictive of TTP in sorafenib treated patients, but also in those receiving TACE	Y- TACE, BSC
(85)	Phase 3 trial	954	VEGF, ANG2, FGF 19, 21,23	VEGF, ANG2, FGF21 predictive of OS. FGF21 predictive of differential OS between sorafenib and lenvatinib	Y-lenvatinib
(86)	Cohort study	78	IGF-1	Adding IGF-1 levels to CP calculation increased prediction of OS	N
(87)	Cohort study	48	18 cytokines	Increase in IL-8 and TNF-a predicts progression	N
(88)	Phase 3 trial-SHARP	602	Ang2, EGF, bFGF, VEGF, sVEGFR-2, sVEGFR-3, HGF, and s-c-KIT IGF-2 circulating Ras	None. High s-c-KIT or low HGF (P of interaction =0.081 and 0.073, respectively)	Y-placebo
(89)	Cohort study	91	TGF-B1	High baseline TGFB predicts poor OS and PFS. Not significant on multivariate analysis	N- receive sorafenib alone or with tegafur/ uracil
(90)	Phase 2	83	IGF-1, IGF-2, IGFBP3	IGF-1	N-Combined two trials. One of sorafenib + tegafur, One Bev+cape
(91)	Phase 2	128	IL-6	IL-6 >4.28 pg/mL predicts OS (HR 2.594)	N-Sorafenib +metronomic chemo
(92)	Cohort study	80	VEGF, HIF-1a	Higher VEGF, and HIF-1a predicts poor OS	N
(93)	Cohort study	124	Ang-2, VEGF, PDGFRb, HGF, CD117, LOXL2, bFGF, PIVKA-II	Predictive model including BCLC stage, bFGF, log PIVKA-II, log HGF, etiology, C-index of 0.884 of tumor response	N
(94)	Cohort study	133	CRP	CRP >1 mg/dL predicts OS (HR 3.31), AFP >400 mg/mL predicts OS (HR 2.76)	N
(95)	Cohort study	165	CRP, AFP	CRP <1mg/dl predicts OS (HR 0.51), AFP <200 ng/mL predicts OS (HR 0.45)	N
(96)	Cohort study	39	EGF, bFGF, HGF, IFN-γ, IL-10, IL-12, IL-2, IL-4, IL-5, IL-6, IL-8, IP-10, MIG, PDGF-BB, SCF, SDF1, TGF-β, TGF-α, TNF-α, and VEGF-A	Elevated IL-5, IL-8, CXCL9, PDGF-BB, TGF-α, and VEGF-A were associated with improved OS in sorafenib but not in hepatic artery infusional chemotherapy	Y- hepatic artery infusional chemotherapy
(97)	Cohort study	97	LDH	Decrease in LDH predicts OS, TTP	N
(98)	Cohort study	44	Lipidomic analysis	phosphatidylcholine (PC)[34:2], PC[34:3]a, PC[35:2], PC[36:4]a, PC[34:3e], acylcarnitine (Car)[18:0], cholesterol ester[20:2], and diacylglycerol (DG)[34:2] predicts response	N
(99)	Cohort study	34	EGF, FGF-2, G-CSF, IFN-γ, IL-12p70, IL-8, IL-17A, IP-10, MCP-1, TNF-α, and VEGF	IL-17A >1.94pg/mL was predictive of PFS (HR 19.96), FGF-2 <20.57pg/mL was predictive of OS (HR 3.24)	N
(100)	Cohort study	115	124 proteins	CDSL, IGJ, LGALS3BP were predictive of sorafenib response (c-index >0.95) and not predictive of TACE response	Y-TACE
(101)	Cohort study	55	VEGF, amphiregulin	Decrease in amphiregulin level was associated with improved OS (HR 0.208)	N
(102)	Cohort study	120	Ang-2, FST, G-CSF, HGF, Leptin, PDGF-BB, PECAM-1, and VEGF (s)-c-KIT	Ang-2 predicts OS (HR 1.95) and PFS, more than 3 cytokines elevated predicts OS	N
(103)	Cohort study	90	FST, G-CSF, HGF, Leptin, PDGF-BB, PECAM-1, Ang-2, VEGF	High Ang2 HR 2.06, and high HGF HR 2.08 were associated with poor OS	N
(104)	Cohort study	63	VEGF levels	VEGF decrease >5% at 8 weeks predicts OS (OR 10 for 1 year survival)	N
(105)	Phase 3 trial	494	VEGFC, heregulin, soluble KIT EPGN and IGF2, VEGFA, HGF, amphiregulin, betacellulin, EGF, epiregulin, hbEGF, TGFα, BFGF, and PDGF-BB	HGF (HR 1.7), VEGFA (HR 1.4), KIT (HR 0.75) predict OS, and VEGFC (HR 0.6) EPGN	N- half of patients received additional erlotinib
(106)	Metanalysis	1202	VEGF	High VEGF HR 1.85 for OS. VEGF SNP associated with OS	N
miRNA					
(107)	Cohort study	20	miR-17-5p, miR-18a, miR-21, miR-34a, miR-122, miR-195, miR-210, miR-214, miR-221, miR-222, miR-223, miR-224, miR-140, miR-328	miR-224 predictive of PFS and OS	N
(108)	Cohort study	93	miR-221	Lower baseline miR-221 predicts response	N
(109)	Cohort study	16	5 miRNAs	miR-181a-5p predicts OS (HR 0.267)	N
(110)	Cohort study	64	522 miRNA from tissue	miR-425-3p predicts PFS	N
(111)	Cohort study	24	miR-18a, miR-21, miR-139-5p, miR-221, miR-224, and miR-10b-3p	High baseline miR-10b-3p Predicts OS (HR 0.522) Not significant on multivariate testing	N
SNPs					
(24)	Cohort study	148	VEGF-A, VEGF-C and VEGFR-1,2,3	*SNPs VEGF-A* rs2010963 and *VEGF-C* rs4604006 predicts OS (HR 0.28, 0.25 respectively) and PFS on multivariate analysis	N
(25)	Cohort study	78	VEGFR2 (KDR) 18 SNPs	*VEGFR2* rs1870377-AA (HR: 0.35) and rs2071559-CC (HR: 2.25) predict OS on multivariate analysis	N
(22)	Cohort study	128	eNOS polymorphisms	eNOS haplotype HT1: T-4b at eNOS-786/eNOS VNTR predicts OS on multivariate analysis (HR 7.03)	N
(23)	Cohort study	135	Ang-2, NOS3 SNPs	*ANGPT2* (Ang2 gene) rs55633437 predicts OS (HR 5.48), NOS3 rs2070744 predicts OS (HR 0.67) on multivariate analysis	N
(112)	Cohort study	210	HIF-1α SNPs	*HIF-1α* rs12434438 no effect measure reported	N
(113)	Cohort study	47	ABCB1 (rs2032582; rs1045642) and ABCG2 (rs2231137; rs2231142; rs2622604	ABCB1 3435C>T, ABCG2 34G>A, ABCG2 1143C>T and ABCG2 421C>A. Trend towards prediction of progression. Not significant	N
(114)	Cohort study	174	whole-genome analysis	SLC15A2 rs2257212 Predicts PFS (HR 2.18)	N

Studies identified by literature review assessing the prognostic ability of biomarkers in patients with advanced HCC treated with sorafenib against clinically relevant endpoints (either overall response rate, disease control rate, PFS or OS) with a statistically significant result. CPI, checkpoint inhibitor; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; miRNA, micro RNA; DCR, disease control rate; PFS, progression-free survival; OS, overall survival; CSC, cancer stem cell; ctDNA, circulating free DNA; CAN, copy number alteration; TTP, time to progression; HR, hazard ratio; IHC, immunohistochemistry; CTC, circulating tumor cell; PBMC, peripheral blood mononuclear cells; ROS, reactive oxygen species; SII, systemic immune inflammation index; NLR, neutrophil lymphocyte ratio; AFP, alpha-fetoprotein; MDSC, myeloid derived suppressor cell; CEC, circulating endothelial cell; CEP, circulating endothelial progenitor; RDW, red cell distribution width; MLR, mixed lymphocyte reaction; CP, Child Pugh; SNP, single nucleotide polymorphism; TACE, trans arterial chemoembolization; CRP, C reactive protein; HCV, hepatitis C virus; HCC, hepatocellular carcinoma.

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Table S2 Literature search results of candidate SNPS

Gene	SNP	Reference
ICAM1	rs1799969 (G241R), rs5498 (K469E)	(115-117)
IL1B	rs1143627 (IL1b- 31 T/C), rs16944 (-511T)	(118-120)
ILA	rs17561, rs143634, rs1800587, rs1143627	(121,122)
IL2	rs2069762 (-330A>C)	(123,124)
IL4	rs2243250, rs2070874	(125,126)
IL 6	rs1800795	(127,128)
IL 8	rs4073 (-251), rs2227306	(129,130)
IL 10	rs3024505, rs1800896, rs3024505, rs1800872 (IL -59)	(131,132)
IL12	rs3212227	(133,134)
IL13	rs20541	(135,136)
IL 17	rs2275913	(137,138)
Mcp-1	rs1024611 (A2518G)	(139)
STAT3	rs3816769	(140,141)
nfkb	rs28362491	(142,143)
TNFa	rs1800629 (-308 G->A)	(144,145)
TGFB	rs1800469	(146,147)
CCL22	rs4359426	(148,149)
iNOS	rs2297518	(150,151)
MMP 1	rs1799750	(152-158)
MMP 7	rs11568818	
MMP 9	rs17576	
MMP 12	rs2276109	
PDL1/PD1	rs11568821, rs11568821 (pd1.3), rs10204525 (pd1.6)	(159,160)
CTLA4 (CD80)	rs231775	(161)
TIM3	rs1036199	(162)
Foxp3	rs3761548, rs2232365	(19,163,164)

No results were found for the following genes: *VCAM1*, *EDNRA/B*, *EMAP2*, *Ang2*, *Tie2*, *IL-5*, *IL18*, *M-CSF (csf1)*, *CSFR1*, *Sdf-1*, *Sema3a*, *NRP1*, *GCSF*, *GM-CSF*, *IFNa*, *OncostatinM*, *CCL2-5*, *CCR2*, *CXCL1-5*, *CXCL8-10*, *CXCL12*, *CXCL17*, *CCL11*, *CCL15*, *CCL28*, *CXCR3*, *CXCR4*, *Bv8*, *ARG1*, *IRF8*, *LAG3*, *ICOS*, *GITR*, *Galectin9*, *CD25.* Candidate SNPs with functional activity identified from literature review of the immune signaling pathways of the HCC tumor immune microenvironment. SNP, single nucleotide polymorphism; HCC, hepatocellular carcinoma.

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Table S3 Chromosomal locations, positions and biological effects of investigated SNPs

SNP ID	Gene	Chr.	Gene position/ effect	Codon exchange	Aa. exchange
rs4604006	*VEGFC*	4	Intron variant		
rs2010963	*VEGFA*	6	5 prime UTR		
rs2070744	*NOS3*	7	Upstream transcript variant		
rs1799983	*NOS3*	7	Missense variant	GAT>GAA	D>E
rs55633437	*ANGPT2*	8	Synonymous variant		
rs1870377	*VEGFR2*	4	Missense Variant	CAA>CAT	Q>H
rs2071559	*VEGFR2*	4	Upstream Variant		
rs10204525	*PDCD1*	2	3 Prime UTR Variant		
rs1024611	*CCL2*	17	5 prime UTR		
rs1036199	*TIM-3*	5	Missense	CGG>CTG	R>L
rs1143627	*IL1B*	2	5 Prime UTR Variant		
rs1143634	*IL1B*	2	Synonymous Variant		
rs11568818	*MMP7*	11	Upstream variant		
rs11568821	*PDCD1*	2	Intron variant		
rs16944	*IL1B*	2	Upstream variant		
rs17561	*IL1A*	2	Missense variant	GCA>TCA	A>S
rs17576	*MMP9*	20	Missense variant	CAG>CCG	Q>L
rs1799750	*MMP1*	11	Upstream variant		
rs1799969	*ICAM1*	19	Missense variant	GGG>AGG	G>R
rs1800469	*TGFB1*	19	Upstream variant		
rs1800587	*IL1A*	2	Upstream variant		
rs1800629	*TNF*	6	Upstream variant		
rs1800795	*IL6*	7	Intron variant		
rs1800872	*IL19*	1	Intron variant		
rs1800896	*IL19*	1	Intron variant		
rs20541	*IL13*	5	Missense variant	CAG>CCG	Q>P
rs2069762	*IL2*	4	Upstream variant		
rs2070874	*IL4*	5	5 Prime UTR Variant		
rs2227306	*CXCL8*	4	Intron variant		
rs2232365	*FOXP3*	X	Intron variant		
rs2243250	*IL4*	5	Upstream variant		
rs2275913	*IL17A*	6	Upstream variant		
rs2276109	*MMP12*	11	Upstream variant		
rs2297518	*NOS2*	17	Missense variant	TCG>TTG	S>L
rs231775	*CTLA4*	2	Missense variant	ACC>GCC	T>A
rs28362491	*NFKB1*	4	Upstream variant		
rs3024505	*IL10*	1	Downstream variant		
rs3212227	*IL12B*	5	3 Prime UTR Variant		
rs3761548	*FOXP3*	X	Intron variant		
rs3816769	*STAT3*	17	Intron variant		
rs4073	*CXCL8*	4	Upstream variant		
rs4359426	*CCL22*	16	Missense variant	GAT>GCT	D>A
rs5498	*ICAM1*	19	Missense variant	AAG>GAG	K>E

UTR, untranslated region; SNP, single nucleotide polymorphism.

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Table S4 Genotype frequencies in study population and general population as well as deviation from Hardy Weinberg Equilibrium

SNP	Genotypes (%)	MAF	HWE
Validation SNPs			
rs4604006	CC/CT/TT (43/44/13)	General population T=0.46 Study population T=0.35	P=0.397840
rs2010963	CC/CG/GG (12/47/41)	General population C=0.37 Study population C=0.37	P=0.635833
rs2070744	CC/CT/TT (11/36/52)	General population C=0.35 Study population C=0.29	P=0.033202
rs1799983	GG/GT/TT (62/31/7)	General population T=0.31 Study population T=0.22	P=0.119858
rs55633437	CC/CA/AA (89/10/1)	General population A=0.04 Study population A=0.07	P=0.250854
rs1870377	AA/AT/TT (11/38/51)	General population A=0.24 Study population A=0.30	P=0.145978
rs2071559	GG/GA/AA (27/41/31)	General population G=0.5 Study population G=0.46	P=0.005423
Exploratory SNPs			
rs10204525	TT/TC/CC (13/31/54)	General population T=0.16 Study population T=0.30	P=0.000274
rs1024611	AA/AG/GG (49/35/16)	General population G=0.28 Study population G=0.33	P=0.000283
rs1036199	CC/CA/AA (2/18/80)	General population C=0.17 Study population C=0.11	P=0.522681
rs1143634	GG/GA/AA (74/22/4)	General population A=0.23 Study population A=0.15	P=0.014409
rs11568818	CC/CT/TT (16/37/47)	General population C=0.44 Study population C=0.33	P=0.004298
rs11568821	CC/CT/TT (87/12/1)	General population T=0.07 Study population T=0.07	P=0.148283
rs16944	GG/GA/AA (36/48/16)	General population A=0.36 Study population A=0.40	P=0.999253
rs17561	CC/CA/AA (65/28/7)	General population A=0.29 Study population A=0.21	P=0.018087
rs17576	AA/AG/GG (34/36/31)	General population G=0.36 Study population G=0.48	P=0.000002
rs1799750	CC/C.DEL/DEL.DEL (32/49/19)	General population DEL=0.49 Study population DEL=0.44	P=0.915367
rs1799969	AA/AG/GG (1/11/88)	General population A=0.1 Study population A=0.07	P=0.340442
rs1800469	GG/GA/AA (36/46/18)	General population A=0.23 Study population A=0.41	P=0.341747
rs1800587	AA/AG/GG (7/29/64)	General population A=0.28 Study population A=0.22	P=0.036665
rs1800629	AA/AG/GG (1/21/77)	General population A=0.15 Study population A=0.12	P=0.644290
rs1800795	CC/CG/GG (8/33/59)	General population C=0.36 Study population C=0.25	P=0.090537
rs1800872	GG/GT/TT (42/41/17)	General population T=0.29 Study population T=0.37	P=0.032667
rs1800896	CC/CT/TT (12/39/49)	General population C=0.45 Study population C=0.32	P=0.107408
rs20541	AA/AG/GG (10/40/50)	General population A=0.21 Study population A=0.3	P=0.447726
rs2069762	CC/CA/AA (15/43/41)	General population C=0.29 Study population C=0.36	P=0.257217
rs2070874	CC/CT/TT (51/26/23)	General population T=0.17 Study population T=0.36	P=0.000000
rs2227306	CC/CT/TT (45/44/11)	General population T=0.36 Study population T=0.33	P=0.784191
rs2232365	CC/CT/TT (51/8/41)	General population T=0.39 Study population T=0.45	P=0.000000
rs2243250	CC/CT/TT (50/25/24)	General population T=0.19 Study population T=0.37	P=0.000000
rs2275913	GG/GA/AA (39/44/17)	General population A=0.33 Study population A=0.39	P=0.169334
rs2276109	TT/TC/CC (86/12/2)	General population C=0.07 Study population C=0.08	P=0.000623
rs2297518	GG/GA/AA (74/24/2)	General population A=0.19 Study population A=0.14	P=0.971112
rs231775	AA/AG/GG (31/49/20)	General population G=0.37 Study population G=0.44	P=0.763588
rs28362491	ATTG.ATTG/ATTG.DEL/DEL.DEL (40/46/15)	General population DEL=0.42 Study population DEL=0.37	P=0.632494
rs3024505	GG/GA/AA (79/21/0)	General population A=0.14 Study population A=0.11	P=0.164468
rs3212227	GG/GT/TT (15/35/50)	General population G=0.22 Study population G=0.32	P=0.000718
rs3761548	GG/GT/TT (58/7/35)	General population T=0.25 Study population T=0.39	P=0.000000
rs3816769	CC/CT/TT (17/44/39)	General population C=0.33 Study population C=0.39	P=0.242653
rs4073	TT/TA/AA (37/44/19)	General population T=0.49 Study population T=0.59	P=0.191191
rs4359426	CC/CA/AA (85/14/1)	General population A=0.05 Study population A=0.08	P=0.755725
rs5498	GG/GA/AA (16/44/40)	General population G=0.43 Study population G=0.38	P=0.247505

SNP, single nucleotide polymorphism.

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Table S5 Univariable analysis and statistical significance of clinical variables against PFS and OS in sorafenib treated patients

Variable	Categories	PFS			OS		
		Median (months)	Hazard ratio (95% CI)	P value*	Median (months)	Hazard ratio (95% CI)	P value*
Age	≥66.5	5.5	0.83 (0.61-1.14)	0.25	18.5	0.77 (0.52-1.1)	0.19
	<66.5	4.0			12.6		
Sex	Male	5.3	0.85 (0.56-1.29)	0.45	16.1	0.88 (0.55-1.4)	0.59
	Female	4.1			12.8		
Hepatitis status	HBV positive	4.0	1.25 (0.7-1.79)	0.23	18	0.94 (0.6-1.5)	0.81
	HBV negative	5.5			13.7		
	HCV positive	5.45	0.84 (0.60-1.18)	0.32	15.4	0.94 (0.6-1.4)	0.75
	HCV negative	4.8			14.9		
Child-Pugh	5	5.13	Reference	P=0.88	19.2	Reference	0.02
	6	5.06	1.11 (0.76-1.61)		9.6	1.96 (1.27-3.04)	
	7	5.32	1.27 (0.64-2.51)		11.9	1.74 (0.84-3.6)	
	8		n/a			n/a	
ECOG	0	5.32	Reference	0.24	17.6	Reference	0.26
	1	4.0	1.21 (0.88-1.67)		12.6	1.3 (0.9-1.9)	
	2		n/a			n/a	
BCLC	A	17.5	Reference	0.58	18.0	Reference	0.33
	B	5.3	1.98 (0.56-6.96)		13.4	4 (0.5-31)	
	C	5.1	1.86 (0.57-6.10)		14.9	3.8 (0.5-27)	
T stage	0	4.6	Reference	0.75	43.3	Reference	0.08
	1	3.5	2.27 (0.55-9.27)		23.2	3.00 (0.60-14.93)	
	2	5.3	1.46 (0.61-3.49)		18.1	1.98 (0.60-6.56)	
	3	5.2	1.46 (0.63-3.37)		12.9	3.12 (0.98-10.00)	
	4	2.8	2.06 (0.61-6.83)		11.9	4.55 (1.00-20.64)	
PVT	Yes	5.3	0.90 (0.65-1.23)	0.49	13	1.4 (0.9-2)	0.11
	No	4.8			18		
Extrahepatic disease	Yes	3.9	1.26 (0.92-1.74)	0.14	13.3	1.06 (0.7-1.6)	0.74
	No	5.5			18		
AFP	≥200	3.7	1.11 (0.80-1.54)	0.54	13.0	1.09 (0.7-1.6)	0.67
	<200	5.7			15.4		
NLR	≥3	4.8	1.13 (0.80-1.61)	0.48	12.8	1.3 (0.95-1.6)	0.06
	<3	5.3			18.9		

*, P values for cox proportional hazards model testing. HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, alpha-fetoprotein; NLR, neutrophil-lymphocyte ratio; ECOG, eastern cooperative oncology group; PVT, portal vein thrombosis; PFS, progression-free survival; OS, overall survival.

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Table S6 Multivariable analysis of clinical variables, validation SNPs and exploratory SNPs for Sorafenib treated patients

Variable	PFS, hazard ratio (P value)	OS, hazard ratio (P value)
Gender (male)	0.66 (0.10)	0.58 (0.06)
Child-Pugh Score		
6 *vs.* 5	0.88 (0.57)	1.48 (0.15)
7 *vs.* 5	1.18 (0.66)	2.07 (0.08)
T stage		
2 *vs.* 1	1.35 (0.58)	3.9 (0.08)
3 *vs.* 1	2.21 (0.14)	8.8 (0.007)
4 *vs.* 1	2.90 (0.12)	12.3 (0.008)
Portal vein thrombus	0.63 (0.09)	0.77 (0.42)
AFP	1.04 (0.24)	1.04 (0.23)
NLR	1.10 (0.47)	1.24 (0.19)
SNPs		
rs1870377	1.27 (0.12)	1.02 (0.93)
rs1024611	0.86 (0.28)	0.67 (0.02)
rs1800896	0.76 (0.05)	0.66 (0.02)
rs231775	1.22 (0.20)	1.13 (0.51)
rs28362491	1.33 (0.045)	1.34 (0.11)

PFS, progression-free survival; OS, overall survival; AFP, alpha-fetoprotein; NLR, neutrophil lymphocyte ratio; SNP, single nucleotide polymorphism.

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Table S7 Demographic and clinical characteristics of patients treated with TACE

Characteristic	Categories	Number (total N=147)
Gender, n (%)	Male	121 (82)
	Female	26 (18)
Age, year, median (range)		67.4 (34.6–86.0)
Ethnicity, n (%)	Asian/pacific islander	47 (32)
	Caucasian	79 (54)
	Black	1 (1)
	Latino	4 (2)
	Aboriginal	0
	Other	3 (2)
	Mixed	0
	Missing	13 (9)
Etiology, n (%)	HBV	32 (22)
	HCV	50 (34)
	Alcohol	36 (24)
	NAFLD	24 (16)
	Other	4 (4)
BCLC, n (%)	A	28 (19)
	B	110 (75)
	C	9 (6)
	Missing	0
Serum AFP, n (%)	≥200	41
	<200	105
	Missing	1
Prior therapy, n (%)	Surgical resection	23 (16)
	RFA	65 (44)
	TACE	0
	Radiation	29 (20)
	Transplant	0
Multifocal, n (%)	Yes	123 (84)
	No	24 (16)
PVT, n (%)	Yes	8 (5)
	No	137 (93)
	Missing	2 (2)
Child-Pugh score, n (%)	A5	113 (77)
	A6	31 (21)
	B7	2 (2)
	≥B8	0
Extrahepatic disease, n (%)	Yes	3 (2)
	No	144 (98)
NLR, n (%)	≥3	53
	<3	93
	Missing	1

HBV, hepatitis B virus; HCV, hepatitis C virus; NAFLD, non-alcoholic fatty liver disease; AFP, alpha-fetoprotein; RFA, radiofrequency ablation; TACE, trans arterial chemoembolization; NLR, neutrophil-lymphocyte ratio; PVT, portal vein thrombosis; PFS, progression-free survival; OS, overall survival.

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Table S8 Univariable analysis and statistical significance of clinical variables against PFS and OS in TACE treated patients

Variable	Value	Median PFS (months)	Hazard ratio (95% CI)	P value	Median OS (months)	Hazard ratio (95% CI)	P value
Age	≥67.4	5.5	0.98 (0.96–0.99)	0.03	28.8	0.99 (0.97–1.03)	1
	<67.4	3.9 (P=0.62)			26.2		
Sex	Male	4.5	1.096 (0.71–1.69)	0.7	25.8	1.06 (0.59–1.88)	0.9
	Female	4.5			30.7		
Etiology	HBV positive	4.3	1.01 (0.67–1.53)	1	46.2	0.6 (0.3–1.05)	0.06
	HBV negative	4.7			25.5		
	HCV positive	4.4	0.99 (0.7–1.4)	0.9	25.5	1.2 (0.7–1.9)	0.5
	HCV negative	4.7			29		
Child-Pugh	5	4.7	Reference	0.7	30.3	Reference	0.1
	6	4.2	1.2 (0.78–1.8)		21.0	1.7 (1.009–2.8)	
	7	5.1	1.4 (0.34–5.6)		3.8	1.8 (0.24–12.9)	
ECOG	0	5	Reference	0.4	46.2	Reference	0.1
	1	4.4	0.82 (0.58–1.2)		23.9	1.5 (0.98–2.4)	
	2	3.5	1.2 (0.49–3)		5.5	2.3 (0.32–17.3)	
BCLC	A	6	Reference	0.03	30.7	Reference	0.3
	B	4.1	1.68 (1.08–2.6)		24.3	1.4 (0.84–2.59)	
	C	6.1	0.99 (0.46–2.2)		27.5	1.7 (0.7–4.1)	
PVT	Yes	2.4	1.2 (0.58–2.5)	0.6	21.6	1.8 (0.77–4.1)	0.2
	No	4.7			27.5		
AFP	≥200	4	1.05 (0.98–1.1)	0.2	21.6	1.09 (1.009–1.18)	0.03
	<200	4.7 (P=0.1)			29		
NLR	≥3	3.8	0.8 (0.59–1.2)	0.4	23.5	1.3 (0.82–2.06)	0.3
	<3	4.6 (P=0.03)			30.3		

HBV, hepatitis B virus; HCV, hepatitis C virus; NAFLD, non-alcoholic fatty liver disease; AFP, alpha-fetoprotein; TACE, trans arterial chemoembolization; NLR, neutrophil-lymphocyte ratio; ECOG, Eastern Cooperative Oncology Group; PVT, portal vein thrombosis; PFS, progression-free survival; OS, overall survival.

Table S9 Univariable analysis validation and exploratory SNPs- TACE treated patients

Gene	Genotypes (%)	Median PFS (months)				Median OS (months)			
		Wt.	Het.	Homo.	P value	Wt.	Het.	Homo.	P value
NOS3 rs2070744	CC/CT/TT (15/54/76)	4	4.4	9	0.06, 0.039	28.8	25.8	27.5	0.9, 0.84
TNF rs1800629	AA/AG/GG (2/32/111)	4.5	4.4	4	*0.4, $^{+}$0.48	23.9	45	41	*0.01, $^{+}$0.007
IL-13 rs20541	AA/AG/GG (13/62/70)	5	4.1	3.9	0.4, 0.48	25.5	30.7	45	0.2, 0.048
NFKB rs28362491	ATTG.ATTG/ATTG.DEL/DEL.DEL (60/66/19)	4.1	5.1	4.8	1, 0.78	25.8	24.3	28.8	0.1, 0.044

*, logrank statistical test, $^{+}$, nested LR statistical test. SNP, single nucleotide polymorphism; TACE, trans arterial chemoembolization; PFS, progression-free survival; OS, overall survival; wt, wild type; Het, heterozygous; Homo, homozygous; LR, likelihood ratio.

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Appendix 1 REMARK Checklist for scoring the quality of the study: Marisi G, Petracci E, Raimondi F, *et al. ANGPT2* and *NOS3* Polymorphisms and Clinical Outcome in Advanced Hepatocellular Carcinoma Patients Receiving Sorafenib (23)

Item to be reported		Page no.	Comment
INTRODUCTION			
1	State the marker examined, the study objectives, and any pre-specified hypotheses.	✓	Discusses aim to determine prognostic value of SNPs within defined genes
MATERIALS AND METHODS			
Patients			
2	Describe the characteristics (e.g., disease stage or co-morbidities) of the study patients, including their source and inclusion and exclusion criteria.	✓	Intermediate/advanced HCC treated with sorafenib. Describes recruitment center, eligibility criteria stated
3	Describe treatments received and how chosen (e.g., randomized or rule-based).	✓	Describes all got sorafenib
Specimen characteristics			
4	Describe type of biological material used (including control samples) and methods of preservation and storage.	✓	DNA extracted from whole blood, in EDTA tubes. No description of preservation
Assay methods			
5	Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments, quantitation methods, and scoring and reporting protocols. Specify whether and how assays were performed blinded to the study endpoint.	✓	DNA extracted using QIAamp DNA Minikit, quality control with nanodrop 1000, genotyping on ABI 3130 Genetic Analyzer. Analysis blinded
Study design			
6	State the method of case selection, including whether prospective or retrospective and whether stratification or matching (e.g., by stage of disease or age) was used. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time.	✓	Retrospective, case selection described, 2012-2015, median f/u 8.9mo
7	Precisely define all clinical endpoints examined.	✓	PFS, OS described
8	List all candidate variables initially examined or considered for inclusion in models.	X	No description of candidate variables
9	Give rationale for sample size; if the study was designed to detect a specified effect size, give the target power and effect size.	X	No rationale given
Statistical analysis methods			
10	Specify all statistical methods, including details of any variable selection procedures and other model-building issues, how model assumptions were verified, and how missing data were handled.	✓	Describes using log rank test and cox proportional hazards model. Describes model was built using variables significant on univariable analysis
11	Clarify how marker values were handled in the analyses; if relevant, describe methods used for cutpoint determination.	✓	categorical
RESULTS			
Data			
12	Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, both overall and for each subgroup extensively examined report the numbers of patients and the number of events.	X	
13	Report distributions of basic demographic characteristics (at least age and sex), standard (disease-specific) prognostic variables, and tumor marker, including numbers of missing values.	✓	*Table 1* describes basic characteristics including missing data
Analysis and presentation			
14	Show the relation of the marker to standard prognostic variables.	X	Not shown
15	Present univariable analyses showing the relation between the marker and outcome, with the estimated effect (e.g., hazard ratio and survival probability). Preferably provide similar analyses for all other variables being analyzed. For the effect of a tumor marker on a time-to-event outcome, a Kaplan-Meier plot is recommended.	✓	
16	For key multivariable analyses, report estimated effects (e.g., hazard ratio) with confidence intervals for the marker and, at least for the final model, all other variables in the model.	✓	
17	Among reported results, provide estimated effects with confidence intervals from an analysis in which the marker and standard prognostic variables are included, regardless of their statistical significance.	✓	Includes nonsignificant clinical variables in final model
18	If done, report results of further investigations, such as checking assumptions, sensitivity analyses, and internal validation.	X	Not reported
DISCUSSION			
19	Interpret the results in the context of the pre-specified hypotheses and other relevant studies; include a discussion of limitations of the study.	✓	Describes exisisting basic science research on ANPT2 and NOS3 snps, as well as data on prognostic significance in other cancers, and other snps studied in HCC
20	Discuss implications for future research and clinical value.	X	Does not describe how studies could validate the predictive use of these markers or how could use in clinic

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Appendix 2 REMARK Checklist for scoring the quality of the study: Casadei Gardini A, Marisi G, Faloppi L, *et al.* eNOS polymorphisms and clinical outcome in advanced HCC patients receiving sorafenib: final results of the ePHAS study (22)

Item to be reported		Page no.	Comment
INTRODUCTION			
1	State the marker examined, the study objectives, and any pre-specified hypotheses.	✓	States SNPs of interest and states the aim of investigating the prognostic value
MATERIALS AND METHODS			
Patients			
2	Describe the characteristics (e.g., disease stage or co-morbidities) of the study patients, including their source and inclusion and exclusion criteria.	✓	Describes included stages and that must be refractory to local treatments, describes recruitment centers, inclusion criteria stated
3	Describe treatments received and how chosen (e.g., randomized or rule-based).	X	Only described that all patients received sorafenib
Specimen characteristics			
4	Describe type of biological material used (including control samples) and methods of preservation and storage.	✓	Used whole blood or FFPE. did not describe storage methods
Assay methods			
5	Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments, quantitation methods, and scoring and reporting protocols. Specify whether and how assays were performed blinded to the study endpoint.	✓	Describes kits for processing, QIAmp DNA minikit or Recoverall, DNA quality assessed by Nanodrop 1000, sequencing on 7500 realtime PCR system (applied biosystems). Does not describe blinding
Study design			
6	State the method of case selection, including whether prospective or retrospective and whether stratification or matching (e.g., by stage of disease or age) was used. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time.	✓	Retrospective, no matching, specifies time period of collection, median follow up 50 months
7	Precisely define all clinical endpoints examined.	✓	PFS, OS described
8	List all candidate variables initially examined or considered for inclusion in models.	✓	age, gender, etiology, Barcelona-Clinic Liver Cancer [BCLC] stage, serum α-FP level and MELD score
9	Give rationale for sample size; if the study was designed to detect a specified effect size, give the target power and effect size.	X	Not mentioned
Statistical analysis methods			
10	Specify all statistical methods, including details of any variable selection procedures and other model-building issues, how model assumptions were verified, and how missing data were handled.	X	Does not describe variable selection procedures, just states clinical covariates were included in model. Does not describe assumption verification or missing data
11	Clarify how marker values were handled in the analyses; if relevant, describe methods used for cutpoint determination.	✓	Categorical
RESULTS			
Data			
12	Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, both overall and for each subgroup extensively examined report the numbers of patients and the number of events.	X	
13	Report distributions of basic demographic characteristics (at least age and sex), standard (disease-specific) prognostic variables, and tumor marker, including numbers of missing values.	✓	Includes *Table 1* and mentions missing values
Analysis and presentation			
14	Show the relation of the marker to standard prognostic variables.	X	
15	Present univariable analyses showing the relation between the marker and outcome, with the estimated effect (e.g., hazard ratio and survival probability). Preferably provide similar analyses for all other variables being analyzed. For the effect of a tumor marker on a time-to-event outcome, a Kaplan-Meier plot is recommended.	✓	
16	For key multivariable analyses, report estimated effects (e.g., hazard ratio) with confidence intervals for the marker and, at least for the final model, all other variables in the model.	✓	Gives HR in multivariate model for snps but not for other variables
17	Among reported results, provide estimated effects with confidence intervals from an analysis in which the marker and standard prognostic variables are included, regardless of their statistical significance.	✓	
18	If done, report results of further investigations, such as checking assumptions, sensitivity analyses, and internal validation.	✓	Includes validation cohort of separate patients. Does not mention sensitivity analysis
DISCUSSION			
19	Interpret the results in the context of the pre-specified hypotheses and other relevant studies; include a discussion of limitations of the study.	✓	. Describes one other study of SNPs as biomarkers for HCC, describes basic science research on eNOS. Describes weakness
20	Discuss implications for future research and clinical value.	X	Discussed results as predictive when they are prognostic

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Appendix 3 REMARK Checklist for scoring the quality of the study: Scartozzi M, Faloppi L, Svegliati Baroni G, *et al.* VEGF and VEGFR genotyping in the prediction of clinical outcome for HCC patients receiving sorafenib: the ALICE-1 study (24)

Item to be reported		Page no.	Comment
INTRODUCTION			
1	State the marker examined, the study objectives, and any pre-specified hypotheses.	✓	Criteria for selection and SNP list specified. Hypothesis not clearly stated
MATERIALS AND METHODS			
Patients			
2	Describe the characteristics (e.g., disease stage or co-morbidities) of the study patients, including their source and inclusion and exclusion criteria.	✓	Exclusion criteria not stated
3	Describe treatments received and how chosen (e.g., randomized or rule-based).	✓	Described all patients received sorafenib
Specimen characteristics			
4	Describe type of biological material used (including control samples) and methods of preservation and storage.	✓	HCC tissue blocks or whole blood, preservation method not stated
Assay methods			
5	Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments, quantitation methods, and scoring and reporting protocols. Specify whether and how assays were performed blinded to the study endpoint.	✓	Commercial assay methods specified. Personnel performing tests were blinded
Study design			
6	State the method of case selection, including whether prospective or retrospective and whether stratification or matching (e.g., by stage of disease or age) was used. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time.	✓	Stated dates of collection, and that patients with intermediate-advanced HCC were chosen, retrospectively. Follow up time stated
7	Precisely define all clinical endpoints examined.	✓	PFS, OS defined
8	List all candidate variables initially examined or considered for inclusion in models.	✓	Lists examined variables
9	Give rationale for sample size; if the study was designed to detect a specified effect size, give the target power and effect size.	✓	Calculates sample size based on absence of progression at 6months
Statistical analysis methods			
10	Specify all statistical methods, including details of any variable selection procedures and other model-building issues, how model assumptions were verified, and how missing data were handled.	X	States model was created using variables significant on univariable testing. Does not comment on missing data handling or verification of model assumptions
11	Clarify how marker values were handled in the analyses; if relevant, describe methods used for cutpoint determination.	✓	Categorical variables (snps)
RESULTS			
Data			
12	Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, both overall and for each subgroup extensively examined report the numbers of patients and the number of events.	X	No flow diagram or comment on dropout
13	Report distributions of basic demographic characteristics (at least age and sex), standard (disease-specific) prognostic variables, and tumor marker, including numbers of missing values.	✓	Prognostic variable distribution listed, does not describe missing values
Analysis and presentation			
14	Show the relation of the marker to standard prognostic variables.	X	No association between SNPs and other prognostic variables
15	Present univariable analyses showing the relation between the marker and outcome, with the estimated effect (e.g., hazard ratio and survival probability). Preferably provide similar analyses for all other variables being analyzed. For the effect of a tumor marker on a time-to-event outcome, a Kaplan-Meier plot is recommended.	✓	
16	For key multivariable analyses, report estimated effects (e.g., hazard ratio) with confidence intervals for the marker and, at least for the final model, all other variables in the model.	✓	HR given with p value but no CI
17	Among reported results, provide estimated effects with confidence intervals from an analysis in which the marker and standard prognostic variables are included, regardless of their statistical significance.	X	Only included significant prognostic variables in final model
18	If done, report results of further investigations, such as checking assumptions, sensitivity analyses, and internal validation.	X	Not described
DISCUSSION			
19	Interpret the results in the context of the pre-specified hypotheses and other relevant studies; include a discussion of limitations of the study.	X	Limitations discussed. Did not comment on other studies associating VEGF SNPs with clinical outcomes
20	Discuss implications for future research and clinical value.	✓	Discusses finding prognostic and recommends validation

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Appendix 4 REMARK Checklist for scoring the quality of the study: Zheng YB, Zhan MX, Zhao W, *et al.* The relationship of kinase insert domain receptor gene polymorphisms and clinical outcome in advanced hepatocellular carcinoma patients treated with sorafenib (25)

Item to be reported		Page no.	Comment
INTRODUCTION			
1	State the marker examined, the study objectives, and any pre-specified hypotheses.	✓	Stated marker (KDR polymorphisms) and objective to determine prognostic effects on TTP, OS
MATERIALS AND METHODS			
Patients			
2	Describe the characteristics (e.g., disease stage or co-morbidities) of the study patients, including their source and inclusion and exclusion criteria.	✓	HCC diagnosed by AASLD guidelines, metastatic/locally advanced not curable and received sorafenib. Excluded if medical comorbidities but did not define
3	Describe treatments received and how chosen (e.g., randomized or rule-based).	✓	All patients received sorafenib, dose described and basis for dose adjustments
Specimen characteristics			
4	Describe type of biological material used (including control samples) and methods of preservation and storage.	✓	Peripheral blood in tube with anticoagulant stored at -80c
Assay methods			
5	Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments, quantitation methods, and scoring and reporting protocols. Specify whether and how assays were performed blinded to the study endpoint.	✓	DNA isolated using Qiagen DNA Isolation Kit according to manufacturer. Genotyping was carried out using the iPLEX Gold™ assay on the MassARRAY Platform. PCR protocol described. Genotyping blinded
Study design			
6	State the method of case selection, including whether prospective or retrospective and whether stratification or matching (e.g., by stage of disease or age) was used. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time.	✓	Retrospective, patients admitted to local hospital between Jan 2010 and Mar 2013. Median follow up described
7	Precisely define all clinical endpoints examined.	✓	Described method for assessing response (mRECIST) and defines TTP and OS
8	List all candidate variables initially examined or considered for inclusion in models.	X	
9	Give rationale for sample size; if the study was designed to detect a specified effect size, give the target power and effect size.	X	
Statistical analysis methods			
10	Specify all statistical methods, including details of any variable selection procedures and other model-building issues, how model assumptions were verified, and how missing data were handled.	X	Described the use of log rank testing and CPH model but did not describe model building or verification, missing data
11	Clarify how marker values were handled in the analyses; if relevant, describe methods used for cutpoint determination.	✓	
RESULTS			
Data			
12	Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, both overall and for each subgroup extensively examined report the numbers of patients and the number of events.	X	
13	Report distributions of basic demographic characteristics (at least age and sex), standard (disease-specific) prognostic variables, and tumor marker, including numbers of missing values.	✓	Included in *Table 2*
Analysis and presentation			
14	Show the relation of the marker to standard prognostic variables.	X	
15	Present univariable analyses showing the relation between the marker and outcome, with the estimated effect (e.g., hazard ratio and survival probability). Preferably provide similar analyses for all other variables being analyzed. For the effect of a tumor marker on a time-to-event outcome, a Kaplan-Meier plot is recommended.	✓	*Table 4* shows univariate analyses. Kaplan Meier plots presented
16	For key multivariable analyses, report estimated effects (e.g., hazard ratio) with confidence intervals for the marker and, at least for the final model, all other variables in the model.	✓	*Table 5*
17	Among reported results, provide estimated effects with confidence intervals from an analysis in which the marker and standard prognostic variables are included, regardless of their statistical significance.	X	Not all prognostic variables included in multivariable model
18	If done, report results of further investigations, such as checking assumptions, sensitivity analyses, and internal validation.	X	
DISCUSSION			
19	Interpret the results in the context of the pre-specified hypotheses and other relevant studies; include a discussion of limitations of the study.	✓	Provides an overview of literature describing functional effects of KDR SNPs, descripes limitations
20	Discuss implications for future research and clinical value.	✓	Discusses need for validation given small sample size and that results may help tailor treatment with sorafenib

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