Research Article

JOURNAL OF HEPATOLOGY

Integrative biomarker analyses indicate etiological variations in hepatocellular carcinoma

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Background & Aims: The purpose of this study was to determine whether biomarkers from baseline plasma and archival tissue specimens collected from patients enrolled in the EVOLVE-1 trial – a randomized phase 3 study of everolimus in hepatocellular carcinoma (HCC) – were associated with prognosis, etiology or ethnicity.

Methods: Circulating plasma levels of bFGF, PLGF, VEGF, VEGF-D, c-Kit, collagen IV, sVEGFR1 and VEGFR2 were measured by ELISA (N = 503). Protein levels of IGF-1R, c-Met, mTOR, Tsc2 were assayed by immunohistochemistry (N = 125). Genomic DNA sequencing was conducted on a panel of 287 cancer-related genes (N = 69).

Results: Patients with baseline plasma concentrations of VEGF or sVEGFR1 above the cohort median had significantly shorter overall survival. These plasma biomarkers retained prognostic significance in a multivariate Cox regression model with geographic region, macroscopic vascular invasion and alpha fetoprotein AFP levels. Membranous c-Met protein levels were significantly lower for Asian patients, as well as for hepatitis B viral etiology. The prevalence of genetic changes were similar to previous reports, along with a trend towards higher *PTEN* and *TSC2* mutations among Asians.

Conclusions: The angiogenesis biomarkers VEGF and sVEGFR1 were independent prognostic predictors of survival in patients with advanced HCC. Potential differences in c-Met and mTOR pathway activation between Asian and non-Asian patients should be considered in future clinical trials.

Lay summary: Our study demonstrates that circulating angiogenesis biomarkers can predict the survival outcome in patients with advanced hepatocellular carcinoma independent of the clinical variables. There is etiology and ethnicity variation in molecular pathway activation in hepatocellular carcinoma, which should be considered for future clinical trial design of targeted therapy.

Clinical trial registration number: NCT01035229.

Keywords: Everolimus; mTOR; c-Met; Tsc2; PI-3 kinase pathway; Next-generation sequencing.

Received 9 November 2015; received in revised form 30 March 2016; accepted 17 April 2016; available online 27 April 2016

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Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; HR, hazard ratio; NASH, nonalcoholic steatohepatitis; sVEGFR1, soluble vascular endothelial growth factor receptor 1; sVEGFR2, soluble vascular endothelial growth factor receptor 2.

Introduction

Since the majority of hepatocellular carcinoma (HCC) patients present with advanced disease with no curative surgical treatment, there is an urgent need to identify additional therapies [1]. Although dozens of targeted therapies have been tested in HCC clinical trials, only the multi-kinase inhibitor, sorafenib, has demonstrated survival benefits [2,3]. A proposed modification to clinical trial design would incorporate biomarkers to identify sub-groups of HCC that may have better response to targeted therapies [4–6]. However, biomarker discovery efforts must take into account the substantial transcriptional and genetic heterogeneity of HCC.

Molecular profiling studies of HCC have identified alterations in key signaling pathways, including receptor tyrosine kinases, Wnt/β-catenin, angiogenesis, oxidative stress, and epigenetic modifiers [7]. High serum levels of several angiogenesis biomarkers have been associated with poor prognosis [8,9]. Several RNA profiling studies have converged on at least three major

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pathway signatures: β-catenin activation, cell cycle proliferation, and inflammatory response [10–12]. Whole exome sequencing studies have also surveyed the most frequently mutated genes in HCC, including: *TERT* promoter (60%); the Wnt/*CTNNB1* pathway (36%); *TP53* (29%); and the nucleosome remodeling enzymes, *ARID1A* and *ARID2* (15%) [13–18]. Notably, there are several reports of etiological differences in mutation frequencies. *TP53* mutations are more prevalent, and *CTNNB1* mutations are less prevalent, in Asian cohorts with predominantly hepatitis B virus (HBV) etiology [19–21]. In addition, genetic alterations in mTOR pathway genes are more prevalent among Asian patients [17]. Thus, molecular biomarkers in HCC may need to be examined in the context of etiological and geographic differences.

The EVOLVE-1 trial investigated everolimus, an mTOR inhibitor, in hepatocellular carcinoma after disease progression or intolerance to sorafenib [22]. The initial objective of this biomarker study was to identify potential biomarkers for everolimus efficacy. Since everolimus did not demonstrate efficacy in the EVOLVE-1 trial, we proceeded with exploratory analyses to determine whether pre-specified biomarkers were associated with prognosis, etiology or ethnicity of HCC patients. A wide panel of assays was chosen to measure various hallmarks of HCC pathogenesis, including angiogenesis, receptor tyrosine kinase activation and recurrent oncogenes and tumor suppressors. Angiogenesis was measured by serum protein levels of bFGF, PLGF, VEGF, VEGF-D, c-Kit, Col-IV, soluble VEGFR1 and soluble VEGFR2. Receptor tyrosine kinase activation was assayed by immunohistochemistry of tissue biopsy specimens, including IGF-1R, c-Met, mTOR and Tsc2. Genetic alterations in recurrent oncogenes and tumor suppressors was assessed by DNA sequencing. In this study, we performed an integrative analysis of circulating, immunohistochemical and genetic biomarkers among patients with collected specimens and assessed the potential etiological variations of these biomarkers.

Materials and methods

Patients and samples

EVOLVE-1 was an international, double-blind, placebo-controlled phase 3 study for adults (age 18 or older) with Barcelona Clinic Liver Cancer stage B or C HCC who had progressed after, or were intolerable for, sorafenib therapy [22]. The protocol was approved by the appropriate ethics body at each participating center. The study was performed per the good clinical practice guidelines, local regulation, and the ethical principles of the declaration of Helsinki. All patients provided written informed consent for correlative studies. This study was registered with ClinicalTrials.gov (NCT01035229).

Plasma biomarkers

Plasma samples were collected up to 6 h prior to the first dose of everolimus or placebo. Plasma biomarker concentrations were measured by commercially available ELISA kits for: bFGF, placenta growth factor, VEGF and soluble VEGFR1 (catalog #: K15029C, Meso Scale Discovery Rockville, MD); c-Kit and soluble VEGFR2 (cat #K15030C, Meso Scale Discovery, Rockville, MD); collagen type IV (catalog #: KT-035, Kamiya Biomedical Company, Tukwila, WA), and VEGF-D (catalog #: DY622, R&D Systems, Minneapolis, MN), according to the manufacturers' instructions.

Immunohistochemistry

Immunohistochemistry (IHC) on tumor specimens from 125 patients was performed at PhenoPath Laboratories (Seattle, WA). Formalin-fixed, paraffin-embedded archival tumor tissue sections were deparaffinized, rehydrated and blocked endogenous peroxidase activity. Heat induced epitope retrieval was performed in EDTA pH 8.0 with steam for 20 min, and Ultravision V-block (Thermo Fisher Scientific, Waltham, MA) was applied for 5 min at room temperature. The slides were incubated with monoclonal antibody for 40 min at room temperature, and then incubated with Ultravision Polymer (Thermo Fisher Scientific, Waltham, MA) for 15 min at room temperature. After incubation in 3,3′-diaminobenzidine (Dako, Carpinteria, CA) for 10 min, the slides were counterstained with hematoxylin, dehydrated, cleared in xylene, and covered by coverslip. Antibodies used included: anti-IGF-1R clone G11 rabbit monoclonal antibody (1:4 dilution, Ventana catalog #790-43446, Tucson, AZ); anti-c-Met clone SP44 rabbit monoclonal antibody (1:100 dilution, Spring Bioscience catalog #M3442, Pleasanton, CA); anti-mTOR clone 7C10 rabbit monoclonal antibody (1:400 dilution, Cell Signaling Technology catalog #2983, Danvers, MA); anti-TSC2 clone D93F12 rabbit monoclonal antibody (1:1000 dilution, Cell Signaling Technology catalog #4308, Danvers, MA).

Immunohistochemistry images were captured with Aperio ScanScope XT ScanScope Console version 101.0.0.18 (Buffalo Grove, IL) and reviewed by a board-certified pathologist. Immunostaining was reported as the percentage of positive cells at each of the following intensities: 0 to 3+ (0 = negative, 1+ = weak (low level), 2+ = moderate, and 3+ = strong). The H-score was calculated as follows: H-score = [fraction of cells with intensity grade 1 (%)] + [fraction of cells with intensity grade 2 (%) × 2] + [fraction of cells with intensity grade 3 (%) × 3]. Specimens with no tumor present or that failed staining were excluded from subsequent analyses. Representative images are shown in Supplementary Fig. 1.

Genetic sequencing

Genomic DNA was extracted from 69 HCC tumors and 69 matched normal (whole blood) specimens, interrogated with a panel of 287 cancer-related genes at Foundation Medicine (Cambridge, MA). The full list of genes is included in Supplementary Table 4. The median exon coverage was 731× on average (range: 333× to 1070×). Single nucleotide variants were called using a Bayesian method as previously described [23]. In brief, this method discarded sequencing reads with mapping quality <25 and base calls with quality ⩽2. The number of variant reads was compared to empirically-observed error rates at each position, and weighted by the tissue-specific mutation rate. Insertions and deletions were identified by assembly of a de Bruijn graph of partially-mapped reads, as described [23]. Single nucleotide variants and indels were annotated as "known" based on the catalogue of somatic mutations in cancer (COSMIC) database [24], and "likely" based on a deleterious protein-coding effect [23]. Copy number alterations were identified by comparing the depth of coverage for each exon *vs.* a process-matched normal control [23].

Statistical analysis

Statistical analyses were conducted with SAS and R software. For prognostic assessment of plasma biomarkers, samples were assigned into a high and low sub-group, based on the median value for each plasma biomarkers. To assess the prognostic effect of those biomarkers, an unadjusted Cox-proportional hazards model was used to estimate the hazard ratios (HR) as well as their respective 95% confidence intervals (CI) between the biomarker high and low sub-groups, irrespective of treatment arms. Unstratified log-rank test was used to test for significant differences between the survival of the sub-groups. Since the EVOLVE-1 trial biomarker analyses were exploratory in nature, no *p* value adjustments for multiple hypothesis testing were conducted. Enrichment of biomarkers with patient etiology or ethnicity were calculated with the hypergeometric test. For c-Met protein levels by IHC, positive samples were defined as at least 50% of tumor nuclei with ⩾2+ staining, the same as defined in the biomarker analysis of the tivantinib phase 2 trial [25]. The Mann-Whitney *U* rank-based test was used to compare c-Met immunohistochemistry H-scores between patient sub-groups.

Results

Clinicopathological characteristics of patients evaluated for biomarkers

A total of 546 patients were randomized (2:1) in the EVOLVE-1 trial, with 362 receiving everolimus treatment and 182 receiving placebo [22]. Plasma samples at baseline were available for 503